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(57) Abstract

The present invention is directed to a method of assembling genomic maps of an organism's DNA or portions thereof. A library of an organism's DNA is provided where the individual genomic segments or sequences are found on more than one clone in the library. Representations of the genome are created, and nucleic acid sequence information is generated from the representations. The sequence information is analyzed to determine clone overlap from a representation. The clone overlap and sequence information from different representations is combined to assemble a genomic map of the organism. Once the genomic map is obtained, genomic sequence information from multiple individuals can be applied to the map and compared with one another to identify single nucleotide polymorphisms. These single nucleotide polymorphisms can be detected, and alleles quantified, by conducting (1) a global PCR amplification which creates a genome representation, and (2) a ligation detection reaction process whose ligation products are captured by hybridization to a support.

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ACCELERATING IDENTIFICATION OF SINGLE NUCLEOTIDE POLYMORPHISMS AND ALIGNMENT OF CLONES IN GENOMIC SEQUENCING

This application claims the benefit of U.S. Provisional Patent Application Serial No. 60/114,881, filed January 6, 1999.

The present invention was made with funding from National Institutes of Health Grant No. GM38839. The United Stated Government may have certain rights in this invention.

FIELD OF THE INVENTION

The present invention is directed to accelerating identification of single nucleotide polymorphisms and an alignment of clone in genomic sequencing.

BACKGROUND OF THE INVENTION

Introduction to Applications of SNPS

Accumulation of genetic changes affecting cell cycle control, cell differentiation, apoptosis, and DNA replication and repair lead to carcinogenesis (Bishop, J. M., "Molecular Themes In Oncogenesis," Cell, 64(2):235-48 (1991)). DNA alterations include large deletions which inactivate tumor supressor genes, amplification to increase expression of oncogenes, and most commonly single nucleotide mutations or polymorphisms which impair gene expression or gene function or predispose an individual to further genomic instability (Table 1).

Table 1: Genetic Alterations Commonly Found in the Human Genome

Type of Alteration	Possible Causes of Alteration	Possible Consequences of Alteration	Detection of Alteration
Single nucleotide polymorphism (SNP)	Inherited variation Methylation Carcinogens Defective repair genes	Silent: does not alter function Missense: alters gene function Nonsense: truncates gene	DNA sequencing SSCP, DGGE, CDGE Protein truncation Mismatch cleavage
Microsatellite instability (MIN)	Defective DNA repair genes Carcinogens	Frameshift: truncates gene	Microsatellite Analysis
Large deletions	Defective DNA repair genes Defective DNA replication genes Illegitimate recombination Double strand break	Loss of gene function	Loss of heterozygosity CGH SNP analysis
DNA amplifications	Defective DNA repair genes Defective DNA replication genes Illegitimate recombination	Overexpression of gene	Competitive PCR CGH SNP analysis
Others: Methylation, Translocation	Defective methylase genes Double strand break	Gene silencing or overexpression; creation of chimeric protein	Endonuclease digestion PCR, FISH

Rapid detection of germline mutations in individuals at risk and accurate characterization of genetic changes in individual tumors would provide opportunities to improve early detection, prevention, prognosis, and specific treatment. However, genetic detection poses the problem of identifying a predisposing polymorphism in the germline or an index mutation in a pre-malignant lesion or early cancer that may be present at many potential sites in many genes. Furthermore, quantification of allele copy number is necessary to detect gene amplification and deletion. Therefore, technologies are urgently needed that can rapidly detect mutation, allele deletion, and allele amplification in multiple genes. Single nucleotide polymorphisms ("SNP"s) are potentially powerful genetic markers for early detection, diagnosis, and staging of human cancers.

Identification of DNA sequence polymorphisms is the cornerstone of modern genome mapping. Initially, maps were created using RFLP markers (Botstein, D., et al., "Construction Of A Genetic Linkage Map In Man Using Restriction Fragment Length Polymorphisms," Amer. J. Hum. Genet., 32:314-331 (1980)), and later by the more polymorphic dinucleotide repeat sequences (Weber, J. L. et al., "Abundant Class Of Human DNA Polymorphisms Which Can Be Typed Using The Polymerase Chain Reaction,." Amer. J. Hum. Genet., 44:388-396 (1989) and Reed, P. W., et al., "Chromosome-Specific Microsatellite Sets For Fluorescence-

Based, Semi-Automated Genome Mapping," Nat Genet, 7(3): 390-5 (1994)). Such sequence polymorphisms may also be used to detect inactivation of tumor suppressor genes via LOH and activation of oncogenes via amplification. These genomic changes are currently being analyzed using conventional Southern hybridizations, competitive PCR, real-time PCR, microsatellite marker analysis, and comparative genome hybridization (CGH) (Ried, T., et al., "Comparative Genomic Hybridization Reveals A Specific Pattern Of Chromosomal Gains And Losses During The Genesis Of Colorectal Tumors," Genes, Chromosomes & Cancer, 15(4):234-45 (1996), Kallioniemi, et al., "ERBB2 Amplification In Breast Cancer Analyzed By Fluorescence In Situ Hybridization," Proc Natl Acad Sci U S A, 89(12):5321-5 (1992), Kallioniemi, et al., "Comparative Genomic Hybridization: A Rapid New Method For Detecting And Mapping DNA Amplification In Tumors," Semin Cancer Biol, 4(1):41-6 (1993), Kallioniemi, et al., "Detection And Mapping Of Amplified DNA Sequences In Breast Cancer By Comparative Genomic Hybridization," Proc Natl Acad Sci U S A, 91(6):2156-60 (1994), Kallioniemi, et al., "Identification Of Gains And Losses Of DNA Sequences In Primary Bladder Cancer By Comparative Genomic Hybridization," Genes Chromosom Cancer, 12(3):213-9 (1995), Schwab, M., et al., "Amplified DNA With Limited Homology To Myc Cellular Oncogene Is Shared By Human Neuroblastoma Cell Lines And A Neuroblastoma Tumour," Nature, 305(5931):245-8 (1983), Solomon, E., et al., "Chromosome 5 Allele Loss In Human Colorectal Carcinomas," Nature, 328(6131):616-9 (1987), Law, D. J., et al., "Concerted Nonsyntenic Allelic Loss In Human Colorectal Carcinoma," Science, 241(4868):961-5 (1988)., Frye, R. A., et al., "Detection Of Amplified Oncogenes By Differential Polymerase Chain Reaction," Oncogene, 4(9):1153-7 (1989), Neubauer, A., et al., "Analysis Of Gene Amplification In Archival Tissue By Differential Polymerase Chain Reaction," Oncogene, 7(5):1019-25 (1992), Chiang, P. W., et al., "Use Of A Fluorescent-PCR Reaction To Detect Genomic Sequence Copy Number And Transcriptional Abundance," Genome Research, 6(10):1013-26 (1996), Heid, C. A., et al., "Real Time Quantitative PCR," Genome Research, 6(10):986-94 (1996), Lee, H. H., et al., "Rapid Detection Of Trisomy 21 By Homologous Gene Quantitative PCR (HGQ-PCR)," Human Genetics, 99(3):364-7 (1997), Boland, C. R., et al., "Microallelotyping Defines The Sequence And Tempo Of Allelic Losses At

Tumour Suppressor Gene Loci During Colorectal Cancer Progression," Nature Medicine, 1(9):902-9 (1995), Cawkwell, L., et al., "Frequency Of Allele Loss Of DCC, p53, RBI, WT1, NF1, NM23 And APC/MCC In Colorectal Cancer Assayed By Fluorescent Multiplex Polymerase Chain Reaction," Br J Cancer, 70(5):813-8 (1994), and Hampton, G. M., et al., "Simultaneous Assessment Of Loss Of Heterozygosity At Multiple Microsatellite Loci Using Semi-Automated Fluorescence-Based Detection: Subregional Mapping Of Chromosome 4 In Cervical Carcinoma," Proceedings of the National Academy of Sciences of the United States of America, 93(13):6704-9 (1996)). Competitive and real-time PCR are considerably faster and require less material than Southern hybridization, although neither technique is amenable to multiplexing. Current multiplex microsatellite marker approaches require careful attention to primer concentrations and amplification conditions. While PCR products may be pooled in sets, this requires an initial run on agarose gels to approximate the amount of DNA in each band (Reed, P. W., et al., "Chromosome-Specific Microsatellite Sets For Fluorescence-Based, Semi-Automated Genome Mapping," Nat Genet, 7(3): 390-5 (1994), and Hampton, G. M., et al., "Simultaneous Assessment Of Loss Of Heterozygosity At Multiple Microsatellite Loci Using Semi-Automated Fluorescence-Based Detection: Subregional Mapping Of Chromosome 4 In Cervical Carcinoma," Proc. Nat'l. Acad. Sci. USA, 93(13):6704-9 (1996)). CGH provides a global assessment of LOH and amplification, but with a resolution range of about 20 Mb. To improve gene mapping and discovery, new techniques are urgently needed to allow for simultaneous detection of multiple genetic alterations.

Amplified fragment length polymorphism ("AFLP") technology is a powerful DNA fingerprinting technique originally developed to identify plant polymorphisms in genomic DNA. It is based on the selective amplification of restriction fragments from a total digest of genomic DNA.

The original technique involved three steps: (1) restriction of the genomic DNA, i.e. with *Eco*RI and *Mse*I, and ligation of oligonucleotide adapters, (2) selective amplification of a subset of all the fragments in the total digest using primers which reached in by from 1 to 3 bases, and (3) gel-based analysis of the amplified fragments. Janssen, et al., "Evaluation of the DNA Fingerprinting Method AFLP as an New Tool in Bacterial Taxonomy," Microbiology, 142(Pt 7):1881-93

(1996); Thomas, et al., "Identification of Amplified Restriction Fragment Polymorphism (AFLP) Markers Tightly Linked to the Tomato Cf-9 Gene for Resistance to Cladosporium fulvum,". Plant J, 8(5):785-94 (1995); Vos, et al., "AFLP: A New Technique for DNA Fingerprinting," Nucleic Acids Res, 23(21):4407-14 (1995); Bachem, et al., "Visualization of Differential Gene Expression Using a Novel Method of RNA Fingerprinting Based on AFLP: Analysis of Gene Expression During Potato Tuber Development," Plant J, 9(5):745-53 (1996); and Meksem, et al., "A High-Resolution Map of the Vicinity of the R1 Locus on Chromosome V of Potato Based on RFLP and AFLP Markers," Mol Gen Genet, 249(1):74-81 (1995), which are hereby incorporated by reference.

AFLP differs substantially from the present invention because it: (i) uses palindromic enzymes, (ii) amplifies both desired *Eco*RI-*Mse*I as well as unwanted *Mse*I- *Mse*I fragments, and (iii) does not identify both alleles when a SNP destroys a pre-existing restriction site. Further, AFLP does not identify SNPs which are outside restriction sites. AFLP does not, and was not designed to create a map of a genome.

Representational Difference Analysis (RDA) was developed by N. Lisitsyn and M. Wigler to isolate the differences between two genomes (Lisitsyn, et al., "Cloning the Differences Between Two Complex Genomes," Science, 259:946-951 (1993), Lisitsyn, et al., "Direct Isolation of Polymorphic Markers Linked to a Trait by Genetically Directed Representational Difference Analysis," Nat Genet, 6(1):57-63 (1994); Lisitsyn, et al., "Comparative Genomic Analysis of Tumors: Detection of DNA Losses and Amplification," Proc Natl Acad Sci USA, 92(1):151-5 (1995); Thiagalingam, et al., "Evaluation of the FHIT Gene in Colorectal Cancers," Cancer Res, 56(13):2936-9 (1996), Li, et al., "PTEN, a Putative Protein Tyrosine Phosphatase Gene Mutated in Human Brain, Breast, and Prostate Cancer," Science, 275(5308):1943-7 (1997); and Schutte, et al., "Identification by Representational Difference Analysis of a Homozygous Deletion in Pancreatic Carcinoma That Lies Within the BRCA2 Region," Proc Natl Acad Sci USA, 92(13):5950-4 (1995). The system was developed in which subtractive and kinetic enrichment was used to purify restriction endonuclease fragments present in one DNA sample, but not in another. The representational part is required to reduce the complexity of the DNA and

generates "amplicons". This allows isolation of probes that detect viral sequences in human DNA, polymorphisms, loss of heterozygosities, gene amplifications, and genome rearrangements.

The principle is to subtract "tester" amplicons from an excess of "driver" amplicons. When the tester DNA is tumor DNA and the driver is normal DNA, one isolates gene amplifications. When the tester DNA is normal DNA and the driver is tumor DNA, one isolates genes which lose function (i.e. tumor suppressor genes).

A brief outline of the procedure is provided herein: (i) cleave both tester and driver DNA with the same restriction endonuclease, (ii) ligate unphosphorylated adapters to tester DNA, (iii) mix a 10-fold excess of driver to tester DNA, melt and hybridize, (iv) fill in ends, (v) add primer and PCR amplify, (vi) digest ssDNA with mung bean nuclease, (vii) PCR amplify, (viii) repeat steps (i) to (vii) for 2-3 rounds, (ix) clone fragments and sequence.

RDA differs substantially from the present invention because it: (i) is a very complex procedure, (ii) is used to identify only a few differences between a tester and driver sample, and (iii) does not identify both alleles when a SNP destroys a pre-existing restriction site. Further, RDA does not identify SNPs which are outside restriction sites. RDA does not, and was not designed to create a map of a genome.

The advent of DNA arrays has resulted in a paradigm shift in detecting vast numbers of sequence variation and gene expression levels on a genomic scale (Pease, A. C., et al., "Light-Generated Oligonucleotide Arrays For Rapid DNA Sequence Analysis," Proc Natl Acad Sci U S A, 91(11):5022-6 (1994), Lipshutz, R. J., et al., "Using Oligonucleotide Probe Arrays To Access Genetic Diversity," Biotechniques, 19(3):442-7 (1995), Eggers, M., et al., "A Microchip For Quantitative Detection Of Molecules Utilizing Luminescent And Radioisotope Reporter Groups," Biotechniques, 17(3):516-25 (1994), Guo, Z., et al., "Direct Fluorescence Analysis Of Genetic Polymorphisms By Hybridization With Oligonucleotide Arrays On Glass Supports," Nucleic Acids Res, 22(24):5456-65 (1994), Beattie, K. L., et al., "Advances In Genosensor Research," Clinical Chemistry, 41(5):700-6 (1995), Hacia, J. G., et al., "Detection Of Heterozygous Mutations In BRCA1 Using High Density Oligonucleotide Arrays And Two-Colour Fluorescence Analysis," Nature Genetics,

14(4):441-7 (1996), Chee, M., et al., "Accessing Genetic Information With High-Density DNA Arrays," Science, 274(5287):610-4 (1996), Cronin, M. T., et al., "Cystic Fibrosis Mutation Detection By Hybridization To Light-Generated DNA Probe Arrays," Hum Mutat, 7(3):244-55 (1996), Drobyshev, A., et al., "Sequence Analysis By Hybridization With Oligonucleotide Microchip: Identification Of Beta-Thalassemia Mutations," Gene, 188(1):45-52 (1997), Kozal, M. J., et al., "Extensive Polymorphisms Observed In HIV-1 Clade B Protease Gene Using High-Density Oligonucleotide Arrays," Nature Medicine, 2(7):753-9 (1996), Yershov, G., et al., "DNA Analysis And Diagnostics On Oligonucleotide Microchips," Proc Natl Acad Sci USA, 93(10):4913-8 (1996), DeRisi, J., et al., "Use Of A CDNA Microarray To Analyse Gene Expression Patterns In Human Cancer," Nature Genetics, 14(4):457-60 (1996), Schena, M., et al., "Parallel Human Genome Analysis: Microarray-Based Expression Monitoring Of 1000 Genes," Proc. Nat'l. Acad. Sci. USA, 93(20):10614-9 (1996), Shalon, D., et al., "A DNA Microarray System For Analyzing Complex DNA Samples Using Two-Color Fluorescent Probe Hybridization," Genome Research, 6(7):639-45 (1996)). Determining deletions, amplifications, and mutations at the DNA level will complement the information obtained from expression profiling of tumors (DeRisi, J., et al., "Use Of A cDNA Microarray To Analyse Gene Expression Patterns In Human Cancer," Nature Genetics, 14(4):457-60 (1996), and Zhang, L., et al., "Gene Expression Profiles In Normal And Cancer Cells," Science, 276:1268-1272 (1997)). DNA chips designed to distinguish single nucleotide differences are generally based on the principle of "sequencing by hybridization" (Lipshutz, R. J., et al., "Using Oligonucleotide Probe Arrays To Access Genetic Diversity," Biotechniques, 19(3):442-7 (1995), Eggers, M., et al., "A Microchip For Quantitative Detection Of Molecules Utilizing Luminescent And Radioisotope Reporter Groups," Biotechniques, 17(3):516-25 (1994), Guo, Z., et al., "Direct Fluorescence Analysis Of Genetic Polymorphisms By Hybridization With Oligonucleotide Arrays On Glass Supports," Nucleic Acids Res, 22(24):5456-65 (1994), Beattie, K. L., et al., "Advances In Genosensor Research," Clinical Chemistry, 41(5):700-6 (1995), Hacia, J. G., et al., "Detection Of Heterozygous Mutations In BRCA1 Using High Density Oligonucleotide Arrays And Two-Colour Fluorescence Analysis," Nature Genetics, 14(4):441-7 (1996), Chee, M., et al., "Accessing Genetic Information With High-

Density DNA Arrays," Science, 274(5287):610-4 (1996), Cronin, M. T., et al., "Cystic Fibrosis Mutation Detection By Hybridization To Light-Generated DNA Probe Arrays," Hum Mutat, 7(3):244-55 (1996), Drobyshev, A., et al., "Sequence Analysis By Hybridization With Oligonucleotide Microchip: Identification Of Beta-Thalassemia Mutations," Gene, 188(1):45-52 (1997), Kozal, M. J., et al., "Extensive Polymorphisms Observed In HIV-1 Clade B Protease Gene Using High-Density Oligonucleotide Arrays," Nature Medicine, 2(7):753-9 (1996), and Yershov, G., et al., "DNA Analysis And Diagnostics On Oligonucleotide Microchips," Proc Natl Acad Sci U S A, 93(10):4913-8 (1996)), or polymerase extension of arrayed primers (Nikiforov, T. T., et al., "Genetic Bit Analysis: A Solid Phase Method For Typing Single Nucleotide Polymorphisms," Nucleic Acids Research, 22(20):4167-75 (1994), Shumaker, J. M., et al., "Mutation Detection By Solid Phase Primer Extension," Human Mutation, 7(4):346-54 (1996), Pastinen, T., et al., "Minisequencing: A Specific Tool For DNA Analysis And Diagnostics On Oligonucleotide Arrays," Genome Research, 7(6):606-14 (1997), and Lockley, A. K., et al., "Colorimetric Detection Of Immobilised PCR Products Generated On A Solid Support," Nucleic Acids Research, 25(6):1313-4 (1997) (See Table 2)). While DNA chips can confirm a known sequence, similar hybridization profiles create ambiguities in distinguishing heterozygous from homozygous alleles (Eggers, M., et al., "A Microchip For Quantitative Detection Of Molecules Utilizing Luminescent And Radioisotope Reporter Groups," Biotechniques, 17(3):516-25 (1994), Beattie, K. L., et al., "Advances In Genosensor Research," Clinical Chemistry, 41(5):700-6 (1995), Chee, M., et al., "Accessing Genetic Information With High-Density DNA Arrays," Science, 274(5287):610-4 (1996), Kozal, M. J., et al., "Extensive Polymorphisms Observed In HIV-1 Clade B Protease Gene Using High-Density Oligonucleotide Arrays," Nature Medicine, 2(7):753-9 (1996), and Southern, E. M., "DNA Chips: Analysing Sequence By Hybridization To Oligonucleotides On A Large Scale," Trends in Genetics, 12(3):110-5 (1996)). Attempts to overcome this problem include using two-color fluorescence analysis (Hacia, J. G., et al., "Detection Of Heterozygous Mutations In BRCA1 Using High Density Oligonucleotide Arrays And Two-Colour Fluorescence Analysis," Nature Genetics, 14(4):441-7 (1996)), 40 overlapping addresses for each known polymorphism (Cronin, M. T., et al., "Cystic

Fibrosis Mutation Detection By Hybridization To Light-Generated DNA Probe Arrays," Hum Mutat, 7(3):244-55 (1996)), nucleotide analogues in the array sequence (Guo, Z., et al., "Enhanced Discrimination Of Single Nucleotide Polymorphisms By Artificial Mismatch Hybridization," Nature Biotech., 15:331-335 (1997)), or adjacent co-hybridized oligonucleotides (Drobyshev, A., et al., "Sequence Analysis By Hybridization With Oligonucleotide Microchip: Identification Of Beta-Thalassemia Mutations," Gene, 188(1):45-52 (1997) and Yershov, G., et al., "DNA Analysis And Diagnostics On Oligonucleotide Microchips," Proc Natl Acad Sci USA, 93(10):4913-8 (1996)). In a side-by-side comparison, nucleotide discrimination using the hybridization chips fared an order of magnitude worse than using primer extension (Pastinen, T., et al., "Minisequencing: A Specific Tool For DNA Analysis And Diagnostics On Oligonucleotide Arrays," Genome Research, 7(6):606-14 (1997)). Nevertheless, solid phase primer extension also generates false positive signals from mononucleotide repeat sequences, template-dependent errors, and template-independent errors (Nikiforov, T. T., et al., "Genetic Bit Analysis: A Solid Phase Method For Typing Single Nucleotide Polymorphisms," Nucl. Acids Res., 22(20):4167-75 (1994) and Shumaker, J. M., et al., "Mutation Detection By Solid Phase Primer Extension," Human Mutation, 7(4):346-54 (1996)).

Over the past few years, an alternate strategy in DNA array design has been pursued. Combined with solution-based polymerase chain reaction/ligase detection assay (PCR/LDR) this array allows for accurate quantification of each SNP allele (See Table 2).

Table 2: Comparison of high-throughput techniques to quantify known SNPs in clinical samples.

Technique	Advantages	Disadvantages
Hybridization on DNA array	1) High density: up to 135,000 addresses. 2) Scan for SNPs in thousands of loci. 3) Detects small insertions/deletions.	1) Specificity determined by hybridization: - difficult to distinguish all SNPs. - difficult to quantify allelic imbalance. 2) Each new DNA target requires a new array.
Mini-sequencing (SNuPE) on DNA array	1) Uses high fidelity polymerase extension: minimizes false positive signal. 2) Potential for single-tube assay.	1) Cannot detect small insertions/deletions. 2) Each new DNA target requires a new array.
PCR/LDR with zip-code capture on universal DNA array	1) Uses high fidelity thermostable ligase; - minimizes false positive signal. 2) Separates SNP identification from signal capture; avoids problems of false hybridization. 3) Quantify gene amplifications and deletions. 4) Universal array works for all gene targets.	1) Requires synthesis of many ligation primers.

For high throughput detection of specific multiplexed LDR products, unique addressable array-specific sequences on the LDR probes guide each LDR product to a designated address on a DNA array, analogous to molecular tags developed for bacterial and yeast genetics genetics (Hensel, M., et al., "Simultaneous Identification Of Bacterial Virulence Genes By Negative Selection," Science, 269(5222):400-3 (1995) and Shoemaker, D. et al., "Quantitative Phenotypic Analysis Of Yeast Deletion Mutants Using A Highly Parallel Molecular Bar-Coding Strategy," Nat Genet, 14(4):450-6 (1996)). The specificity of this reaction is determined by a thermostable ligase which allows detection of (i) dozens to hundreds of polymorphisms in a single-tube multiplex format, (ii) small insertions and deletions in repeat sequences, and (iii) low level polymorphisms in a background of normal DNA. By uncoupling polymorphism identification from hybridization, each step may be optimized independently, thus allowing for quantitative assessment of allele imbalance even in the presence of stromal cell contamination. This approach has the potential to rapidly identify multiple gene deletions and amplifications associated with tumor progression, as well as lead to the discovery of new oncogenes and tumor suppressor genes. Further, the ability to score hundreds to thousands of SNPs has utility in linkage studies (Nickerson, D. A., et al., "Identification Of Clusters Of Biallelic Polymorphic Sequence-Tagged Sites (pSTSs) That Generate Highly Informative And Automatable Markers For Genetic Linkage Mapping," Genomics, 12(2):377-87 (1992), Lin, Z., et al., "Multiplex Genotype Determination At A Large Number Of Gene Loci," Proc Natl Acad Sci USA, 93(6):2582-7 (1996), Fanning, G. C., et al., "Polymerase Chain Reaction Haplotyping Using 3' Mismatches In The Forward And Reverse Primers: Application To The Biallelic Polymorphisms Of Tumor Necrosis Factor And Lymphotoxin Alpha," Tissue Antigens, 50(1):23-31 (1997), and Kruglyak, L., "The Use of a Genetic Map of Biallelic Markers in Linkage Studies," Nature Genetics, 17:21-24 (1997)), human identification (Delahunty, C., et al., "Testing The Feasibility Of DNA Typing For Human Identification By PCR And An Oligonucleotide Ligation Assay," Am. J. Hum. Gen., 58(6):1239-46 (1996) and Belgrader, P., et al., "A Multiplex PCR-Ligase Detection Reaction Assay For Human Identity Testing," Gen. Sci. & Tech., 1:77-87 (1996)), and mapping complex human

diseases using association studies where SNPs are identical by decent (Collins, F. S., "Positional Cloning Moves From Perditional To Traditional," Nat Genet, 9(4):347-50 (1995), Lander, E. S., "The New Genomics: Global Views Of Biology," Science, 274(5287):536-9 (1996), Risch, N. et al., "The Future Of Genetic Studies Of Complex Human Diseases," Science, 273(5281):1516-7 (1996), Cheung, V. G. et al., "Genomic Mismatch Scanning Identifies Human Genomic DNA Shared Identical By Descent," Genomics, 47(1):1-6 (1998), Heung, V. G., et al., "Linkage-Disequilibrium Mapping Without Genotyping," Nat Genet, 18(3):225-230 (1998), and McAllister, L., et al., "Enrichment For Loci Identical-By-Descent Between Pairs Of Mouse Or Human Genomes By Genomic Mismatch Scanning," Genomics, 47(1):7-11 (1998)).

For 85% of epithelial cancers, loss of heterozygosity and gene amplification are the most frequently observed changes which inactivate the tumor suppressor genes and activate the oncogenes. Southern hybridizations, competitive PCR, real time PCR, microsatellite marker analysis, and comparative genome hybridization (CGH) have all been used to quantify changes in chromosome copy number (Ried, T., et al., "Comparative Genomic Hybridization Reveals A Specific Pattern Of Chromosomal Gains And Losses During The Genesis Of Colorectal Tumors," Genes, Chromosomes & Cancer, 15(4):234-45 (1996), Kallioniemi, et al., "ERBB2 Amplification In Breast Cancer Analyzed By Fluorescence In Situ Hybridization," Proc Natl Acad Sci USA, 89(12):5321-5 (1992), Kallioniemi, et al., "Comparative Genomic Hybridization: A Rapid New Method For Detecting And Mapping DNA Amplification In Tumors," Semin Cancer Biol, 4(1):41-6 (1993), Kallioniemi, et al., "Detection And Mapping Of Amplified DNA Sequences In Breast Cancer By Comparative Genomic Hybridization," Proc Natl Acad Sci USA, 91(6):2156-60 (1994), Kallioniemi, et al., "Identification Of Gains And Losses Of DNA Sequences In Primary Bladder Cancer By Comparative Genomic Hybridization," Genes Chromosom Cancer, 12(3):213-9 (1995), Schwab, M., et al., "Amplified DNA With Limited Homology To Myc Cellular Oncogene Is Shared By Human Neuroblastoma Cell Lines And A Neuroblastoma Tumour," Nature, 305(5931):245-8 (1983), Solomon, E., et al., "Chromosome 5 Allele Loss In Human Colorectal Carcinomas," Nature, 328(6131):616-9 (1987), Law, D. J., et al., "Concerted Nonsyntenic Allelic Loss In Human Colorectal Carcinoma," Science,

241(4868):961-5 (1988), Frye, R. A., et al., "Detection Of Amplified Oncogenes By Differential Polymerase Chain Reaction," Oncogene, 4(9):1153-7 (1989), Neubauer, A., et al., "Analysis Of Gene Amplification In Archival Tissue By Differential Polymerase Chain Reaction," Oncogene, 7(5):1019-25 (1992), Chiang, P. W., et al., "Use Of A Fluorescent-PCR Reaction To Detect Genomic Sequence Copy Number And Transcriptional Abundance," Genome Research, 6(10):1013-26 (1996), Heid, C. A., et al., "Real Time Quantitative PCR," Genome Research, 6(10):986-94 (1996), Lee, H. H., et al., "Rapid Detection Of Trisomy 21 By Homologous Gene Quantitative PCR (HGQ-PCR)," Human Genetics, 99(3):364-7 (1997), Boland, C. R., et al., "Microallelotyping Defines The Sequence And Tempo Of Allelic Losses At Tumour Suppressor Gene Loci During Colorectal Cancer Progression," Nature Medicine, 1(9):902-9 (1995), Cawkwell, L., et al., "Frequency Of Allele Loss Of DCC, p53, RBI, WT1, NF1, NM23 And APC/MCC In Colorectal Cancer Assayed By Fluorescent Multiplex Polymerase Chain Reaction," Br J Cancer, 70(5):813-8 (1994), and Hampton, G. M., et al., "Simultaneous Assessment Of Loss Of Heterozygosity At Multiple Microsatellite Loci Using Semi-Automated Fluorescence-Based Detection: Subregional Mapping Of Chromosome 4 In Cervical Carcinoma," Proc. Nat'l. Acad. Sci. USA, 93(13):6704-9 (1996)). Recently, a microarray of consecutive BACs from the long arm of chromosome 20 has been used to accurately quantify 5 regions of amplification and one region of LOH associated with development of breast cancer. This area was previously thought to contain only 3 regions of amplification (Tanner, M. et al., "Independent Amplification And Frequent Co-Amplification Of Three Nonsyntenic Regions On The Long Arm Of Chromosome 20 In Human Breast Cancer," Cancer Research, 56(15):3441-5 (1996)). Although this approach will yield valuable information from cell lines, it is not clear it will prove quantitative when starting with microdissected tissue which require PCR amplification. Competitive and real time PCR approaches require careful optimization to detect 2-fold differences (Frye, R. A., et al., "Detection Of Amplified Oncogenes By Differential Polymerase Chain Reaction," Oncogene, 4(9):1153-7 (1989), Neubauer, A., et al., "Analysis Of Gene Amplification In Archival Tissue By Differential Polymerase Chain Reaction," Oncogene, 7(5):1019-25 (1992), Chiang, P. W., et al., "Use Of A Fluorescent-PCR Reaction To Detect Genomic Sequence Copy Number And Transcriptional

Abundance," Genome Research, 6(10):1013-26 (1996), Heid, C. A., et al., "Real Time Quantitative PCR," Genome Research, 6(10):986-94 (1996), and Lee, H. H., et al., "Rapid Detection Of Trisomy 21 By Homologous Gene Quantitative PCR (HGQ-PCR)," Human Genetics, 99(3):364-7 (1997)). Unfortunately, stromal contamination may reduce the ratio between tumor and normal chromosome copy number to less than 2-fold. By using a quantitative SNP -DNA array detection, each allele can be distinguished independently, thus reducing the effect of stromal contamination in half. Further by comparing the ratio of allele-specific LDR product formed from a tumor to control gene between a tumor and normal sample, it may be possible to distinguish gene amplification from loss of heterozygosity at multiple loci in a single reaction.

Using PCR/LDR to detect SNPs.

The ligase detection reaction ("LDR") is ideal for multiplexed discrimination of single-base mutations or polymorphisms (Barany, F., et al., "Cloning, Overexpression, And Nucleotide Sequence Of A Thermostable DNA Ligase Gene," Gene, 109:1-11 (1991), Barany, F., "Genetic Disease Detection And DNA Amplification Using Cloned Thermostable Ligase," Proc. Natl. Acad. Sci. USA, 88:189-193 (1991), and Barany, F., "The Ligase Chain Reaction (LCR) In A PCR World," PCR Methods and Applications, 1:5-16 (1991)). Since there is no polymerization step, several probe sets can ligate along a gene without interference. The optimal multiplex detection scheme involves a primary PCR amplification, followed by either LDR (two probes, same strand) or ligase chain reaction ("LCR") (four probes, both strands) detection. This approach has been successfully applied for simultaneous multiplex detection of 61 cystic fibrosis alleles (Grossman, P. D., et al., "High-Density Multiplex Detection Of Nucleic Acid Sequences: Oligonucleotide Ligation Assay And Sequence-Coded Separation," Nucleic Acids Res., 22:4527-4534 (1994) and Eggerding, F. A., et al., "Fluorescence-Based Oligonucleotide Ligation Assay For Analysis Of Cystic Fibrosis Transmembrane Conductance Regulator Gene Mutations," Human Mutation, 5:153-165 (1995)), 6 hyperkalemic periodic paralysis alleles (Feero, W. T., et al., "Hyperkalemic Periodic Paralysis: Rapid Molecular Diagnosis And Relationship Of Genotype To Phenotype In 12 Families," Neurology,

43:668-673 (1993)), and 20 21-hydroxylase deficiency alleles (Day, D., et al., "Detection Of Steroid 21 Hydroxylase Alleles Using Gene-Specific PCR And A Multiplexed Ligation Detection Reaction," Genomics, 29:152-162 (1995) and Day, D. J., et al., "Identification Of Non-Amplifying CYP21 Genes When Using PCR-Based Diagnosis Of 21-Hydroxylase Deficiency In Congenital Adrenal Hyperplasia (CAH) Affected Pedigrees," Hum Mol Genet, 5(12):2039-48 (1996)).

21-hydroxylase deficiency has the highest carrier rate of any genetic disease, with 6% of Ashkenazi Jews being carriers. Approximately 95% of mutations causing 21-hydroxylase deficiency are the result of recombinations between an inactive pseudogene termed *CYP21P* and the normally active gene termed *CYP21*, which share 98% sequence homology (White, P. C., et al., "Structure Of Human Steroid 21-Hydroxylase Genes," Proc. Natl. Acad. Sci. USA, 83:5111-5115 (1986)). PCR/LDR was developed to rapidly determine heterozygosity or homozygosity for any of the 10 common apparent gene conversions in *CYP21*. By using allele-specific PCR, defined regions of *CYP21* are amplified without amplifying the *CYP21P* sequence. The presence of wild-type or pseudogene mutation is subsequently determined by fluorescent LDR. Discriminating oligonucleotides complementary to both *CYP21* and *CYP21P* are included in equimolar amounts in a single reaction tube so that a signal for either active gene, pseudogene, or both is always obtained. PCR/LDR genotyping (of 82 samples) was able to readily type compound heterozygotes with multiple gene conversions in a multiplexed reaction, and was in complete agreement with direct sequencing/ASO analysis. This method was able to distinguish insertion of a single T nucleotide into a $(T)_7$ tract, which cannot be achieved by allele-specific PCR alone (Day, D., et al., "Detection Of Steroid 21 Hydroxylase Alleles Using Gene-Specific PCR And A Multiplexed Ligation Detection Reaction," Genomics, 29:152-162 (1995)). A combination of PCR/LDR and microsatellite analysis revealed some unusual cases of PCR allele dropout (Day, D. J., et al., "Identification Of Non-Amplifying CYP21 Genes When Using PCR-Based Diagnosis Of 21-Hydroxylase Deficiency In Congenital Adrenal Hyperplasia (CAH) Affected Pedigrees," Hum Mol Genet, 5(12):2039-48 (1996)). The LDR

approach is a single-tube reaction which enables multiple samples to be analyzed on a single polyacrylamide gel.

A PCR/LDR assay has been developed to detect germline mutations, found at high frequency (3% total), in BRCA1 and BRCA2 genes in the Jewish population. The mutations are: BRCA1, exon 2 185delAG; BRCA1, exon 20 5382insC; BRCA2, exon 11 6174delT. These mutations are more difficult to detect than most germline mutations, as they involve slippage in short repeat regions. A preliminary screening of 20 samples using multiplex PCR of three exons and LDR of six alleles in a single tube assay has successfully detected the three Ashkenazi BRCA1 and BRCA2 mutations.

Multiplexed PCR for amplifying many regions of chromosomal DNA simultaneously.

A coupled multiplex PCR/PCR/LDR assay was developed to identify armed forces personnel. Several hundred SNPs in known genes with heterozygosities > 0.4 are currently listed. Twelve of these were amplified in a single PCR reaction as follows: Long PCR primers were designed to have gene-specific 3' ends and 5' ends complementary to one of two sets of PCR primers. The upstream primers were synthesized with either FAM- or TET-fluorescent labels. These 24 gene-specific primers were pooled and used at low concentration in a 15 cycle PCR. After this, the two sets of primers were added at higher concentrations and the PCR was continued for an additional 25 cycles. The products were separated on an automated ABD 373A DNA Sequencer. The use of these primers produces similar amounts of multiplexed products without the need to carefully adjust gene-specific primer concentrations or PCR conditions (Belgrader, P., et al., "A Multiplex PCR-Ligase Detection Reaction Assay For Human Identity Testing," Genome Science and Technology, 1:77-87 (1996)). In a separate experiment, non-fluorescent PCR products were diluted into an LDR reaction containing 24 fluorescently labeled allele-specific LDR probes and 12 adjacent common LDR probes, with products separated on an automated DNA sequencer. LDR probe sets were designed in two ways: (i) allele-specific FAM- or TET-labeled LDR probes of uniform length, or (ii) allele-specific HEX-labeled LDR probes differing in length by two bases. A comparison of LDR profiles of several

individuals demonstrated the ability of PCR/LDR to distinguish both homozygous and heterozygous genotypes at each locus (Id.). The use of PCR/PCR in human identification to simultaneously amplify 26 loci has been validated (Lin, Z., et al., "Multiplex Genotype Determination At A Large Number Of Gene Loci," Proc Natl Acad Sci USA, 93(6):2582-7 (1996)), or ligase based detection to distinguish 32 alleles although the latter was in individual reactions (Nickerson, D. A., et al., "Identification Of Clusters Of Biallelic Polymorphic Sequence-Tagged Sites (pSTSs) That Generate Highly Informative And Automatable Markers For Genetic Linkage Mapping," Genomics, 12(2):377-87 (1992)). This study validates the ability to multiplex both PCR and LDR reactions in a single tube, which is a prerequisite for developing a high throughput method to simultaneously detect SNPs throughout the genome.

For the PCR/PCR/LDR approach, two long PCR primers are required for each SNP analyzed. A method which reduces the need for multiple PCR primers would give significant savings in time and cost of a large-scale SNP analysis. The present invention is directed to achieving this objective.

SUMMARY OF THE INVENTION

The present invention is directed to a method of assembling genomic maps of an organism's DNA or portions thereof. A library of an organism's DNA is provided where the individual genomic segments or sequences are found on more than one clone in the library. Representations of the genome are created, and nucleic acid sequence information is generated from the representations. The sequence information is analyzed to determine clone overlap from a representation. The clone overlap and sequence information from different representations is combined to assemble a genomic map of the organism.

As explained in more detail *infra,* the representation can be created by selecting a subpopulation of genomic segments out of a larger set of the genomic segments in that clone. In particular, this is achieved by first subjecting an individual clone to a first restriction endonuclease under conditions effective to cleave DNA from the individual clone so that a degenerate overhang is created in the clone.

Non-palindromic complementary linker adapters are added to the overhangs in the presence of ligase and the first restriction endonuclease to select or amplify particular fragments from the first restriction endonuclease digested clone as a representation. As a result, sufficient linker-genomic fragment products are formed to allow determination of a DNA sequence adjacent to the overhang. Although a number of first restriction endonucleases are suitable for use in this process, it is particularly desirable to use the enzyme *DrdI* to create the representation which comprises what are known as *DrdI* islands (i.e. the genomic segments which are produced when *DrdI* cleaves the genomic DNA in the clones).

The procedure is amenable to automation and requires just a single extra reaction (simultaneous cleavage/ligation) compared to straight dideoxy sequencing. Use of from 4 to 8 additional linker adapters/primers is compatible with microtiter plate format for delivery of reagents. A step which destroys the primers after the PCR amplification allows for direct sequencing without purifying the PCR products.

A method is provided for analyzing sequencing data allowing for assignment of overlap between two or more clones. The method deconvolutes singlet, doublet, and triplet sequencing runs allowing for interpretation of the data. For sequencing runs which are difficult to interpret, sequencing primers containing an additional one or two bases on the 3' end will generate a readable sequence. As an alternative to deconvoluting doublet and triplet sequencing runs, other enzymes may be used to create short representational fragments. Such fragments may be differentially enriched via ultrafiltration to provide dominant signal, or, alternatively, their differing length provides unique sequence signatures on a full length sequencing run.

About 200,000 to 300,000 *Drd* Islands are predicted in the human genome. The *DrdI* Islands are a representation of $1/15^{th}$ to $1/10^{th}$ of the genome. With an average BAC size of 100-150 kb, a total of 20,000 to 30,000 BAC clones would cover the human genome, or 150,000 clones would provide 5-fold coverage. Using the *DrdI* island approach, 4-6 sequencing runs are required for a total of 600,000 to 900,000 sequencing reactions. New automated capillary sequencing machines (Perkin Elmer 3700 machine) can run 2,304 short (80-100bp) sequencing

reads per day. Thus, the *DrdI* approach for overlapping all BAC clones providing a 5-fold coverage of the human genome would require only 39 days using 10 of the new DNA sequencing machines.

The above approach will provide a highly organized contig of the entire genome for just under a million sequencing reactions, or about $1/70^{th}$ of the effort required by just random clone overlap. Subsequently, random sequencing will fill in the sequence information between *DrdI* islands. Since the islands are anchored in the contig, this will result in a 2- to 4-fold reduction in the amount of sequencing necessary to obtain a complete sequence of the genome.

Single nucleotide polymorphisms or SNPs have been proposed as valuable tools for gene mapping and discovering genes associated with common diseases. The present invention provides a rapid method to find mapped single nucleotide polymorphisms within genomes. A representation of the genomes of multiple individuals is cloned into a common vector. Sequence information generated from representational library is analyzed to determine single nucleotide polymorphisms.

The present invention provides a method for large scale detection of single nucleotide polymorphisms ("SNP"s) on a DNA array. This method involves creating a representation of a genome from a clinical sample. A plurality of oligonucleotide probe sets are provided with each set characterized by (a) a first oligonucleotide probe, having a target-specific portion and an addressable array-specific portion, and (b) a second oligonucleotide probe, having a target-specific portion and a detectable reporter label. The oligonucleotide probes in a particular set are suitable for ligation together when hybridized adjacent to one another on a corresponding target nucleotide sequence, but have a mismatch which interferes with such ligation when hybridized to any other nucleotide sequence present in the representation of the sample. A mixture is formed by blending the sample, the plurality of oligonucleotide probe sets, and a ligase. The mixture is subjected to one or more ligase detection reaction ("LDR") cycles comprising a denaturation treatment, where any hybridized oligonucleotides are separated from the target nucleotide sequences, and a hybridization treatment, where the oligonucleotide probe sets hybridize at adjacent positions in a base-specific manner to their respective target

nucleotide sequences, if present in the sample, and ligate to one another to form a ligation product sequence containing (a) the addressable array-specific portion, (b) the target-specific portions connected together, and (c) the detectable reporter label. The oligonucleotide probe sets may hybridize to nucleotide sequences in the sample other than their respective target but do not ligate together due to a presence of one or more mismatches and individually separate during the denaturation treatment. A solid support with different capture oligonucleotides immobilized at particular sites is provided where the capture oligonucleotides have nucleotide sequences complementary to the addressable array-specific portions. After subjecting the mixture to one or more ligase detection reaction cycles, the mixture is contacted with the solid support under conditions effective to hybridize the addressable array-specific portions to the capture oligonucleotides in a base-specific manner. As a result, the addressable array-specific portions are captured on the solid support at the site with the complementary capture oligonucleotide. Finally the reporter labels of ligation product sequences captured to the solid support at particular sites are detected which indicates the presence of single nucleotide polymorphisms.

It has been estimated that 30,000 to 300,000 SNPs will be needed to map the positions of genes which influence the major multivariate diseases in defined populations using association methods. Since the above SNP database is connected to a closed map of the entire genome, new genes may be rapidly discovered. Further, the representative PCR/ LDR / universal array may be used to quantify allele imbalance. This allows for use of SNPs to discover new tumor suppressor genes, which undergo loss of heterozygosity, or oncogenes, which undergo amplification, in various cancers.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic drawing showing the sequencing of *DrdI* islands in random plasmid or cosmid clones in accordance with the present invention.

Figure 2 is a schematic drawing of a first embodiment for sequencing restriction enzyme generated representations.

Figure 3 is a schematic drawing of a second embodiment for sequencing restriction enzyme generated representations.

Figure 4 is a schematic drawing for DNA sequencing directly from PCR amplified DNA without primer interference.

Figure 5 is a schematic drawing showing another embodiment of the *DrdI* island sequencing technique of the present invention.

Figure 6 is a schematic drawing showing a further alternative embodiment of sequencing *DrdI* islands in random BAC clones using PCR amplification.

Figure 7 shows the three degrees of specificity in amplifying a *DrdI* representation.

Figure 8 shows the *DrdI* and *BglI* site frequencies per 40kb in the Met Oncogene BAC from the 7q31 chromosome. The locations of the 12 *DrdI* and 16 *BglI* sites in a 171,905 bp clone are shown pictorially and in tabular form, indicating the type of overhang and the complement to that overhang. For this clone, per 40 kb, the unique sites (i.e.singlets) are as follows: 1.4 of such unique *DrdI* sites and 3.3 of such unique *BglI* sites. In this clone, per 40 kb, the sites with the 3'overhang having the same last 2 bases -- doublets (i.e. *) are as follows: 1.0 of such *DrdI* sites and 4.3 of such *BglI* sites. The number of palindromic overhangs not used (i.e. ^) is as follows: 2 overhangs for *DrdI* and 0 overhangs for *BglI*. The number of sites with the 3' overhang having the same last 2 bases within the BAC clone used exactly once—singlets (i.e. @) is as follows: 2 of such *DrdI* sites and 5 of such *BglI* sites. The number of sites with the 3' overhang having the same last 2 bases within the BAC clone used exactly twice—doublets (i.e. #) is as follows: 4 of such *DrdI* sites and 5 of such *BglI* sites. The number of sites with the 3' overhang having the same last 2 bases within the BAC clone used more than twice (i.e. X) is as follows: 0 of such *DrdI* sites and 3 of such *BglI* sites.

Figure 9 shows the *SapI* site frequencies per 40kb in the Met Oncogene BAC from the 7q31 chromosome. The locations of the 25 *SapI* sites in a 171,905 bp clone are shown pictorially and in tabular form, indicating the type of overhang and the complement to that overhang. The number of sites with the 3' overhang having the same last 2 bases within the BAC clone used exactly once—singlets (i.e. @) is 5

of such *Sap*I sites. The number of sites with the 3' overhang having the same last 2 bases within the BAC clone used exactly twice—doublets (i.e. #) is 10 of such *Sap*I sites. The number of sites with the 3' overhang having the same last 2 bases within the BAC clone used more than twice (i.e. X) is 3 of such *Sap*I sites.

Figure 10 shows the *Drd*I and *Bgl*I site frequencies per 40kb in the HMG Oncogene BAC from the 7q31 chromosome. The locations of the 11 *Drd*I and 12 *Bgl*I sites in a 165,608 bp clone are shown pictorially and in tabular form, indicating the type of overhang and the complement to that overhang. For this clone, per 40 kb, the unique sites (i.e. singlets) are as follows: 1.2 of such unique *Drd*I sites and 3.9 of such unique *Bgl*I sites. In this clone, per 40 kb, the sites with the 3' overhang having the same last 2 bases--doublets (i.e. *) are as follows: 1.2 of such *Drd*I sites and 2.0 of such *Bgl*I sites. The number of palindromic overhangs not used (i.e. ^) is as follows: 1 overhang for *Drd*I and 0 overhangs for *Bgl*I. The number of sites with the 3' overhang having the same last 2 bases within the BAC clone used exactly once—singlets (i.e. @) is as follows: 3 of such *Drd*I sites and 5 of such *Bgl*I sites. The number of sites with the 3' overhang having the same last 2 bases within the BAC clone used exactly twice—doublets (i.e. #) is as follows: 2 of such *Drd*I sites and 4 of such *Bgl*I sites. The number of sites with the 3' overhang having the same last 2 bases within the BAC clone used more than twice (i.e. X) is as follows: 1 of such *Drd*I sites and 3 of such *Bgl*I sites.

Figure 11 shows the *Sap*I site frequencies per 40kb in the HMG Oncogene BAC from the 7q31 chromosome with the locations of the 12 *Sap*I sites in a 165,608 bp clone being shown in pictorial and tabular form, indicating the type of overhang and the complement to that overhang. The number of sites with the 3' overhang having the same last 2 bases within the BAC clone used exactly once—singlets (i.e. @) is 4 of such *Sap*I sites. The number of sites with the 3' overhang having the same last 2 bases within the BAC clone used exactly twice—doublets (i.e. #) is 1 of such *Sap*I sites. The number of sites with the 3' overhang having the same last 2 bases with BAC in the clone used more than twice (i.e. X) is 2 of such *Sap*I sites.

Figure 12 shows the *Drd*I and *Bgl*I site frequencies per 40kb in the Pendrin Oncogene BAC from the 7q31 chromosome with the locations of the 10 *Drd*I

and 17 *BglI* sites in a 97,943 bp clone being shown in pictorial and tabular form, indicating the type of overhang, and the complement to that overhang. For this clone, per 40 kb, the unique sites are as follows: 1.3 of such unique *DrdI* sites and 5.0 of such unique *BglI* sites. In this clone, per 40 kb, the sites with the 3' overhang having the same last 2 bases--doublets (i.e. *) are as follows: 2.1 of such *DrdI* sites and 9.2 of such *BglI* sites. The number of palindromic overhangs not used (i.e. ^) is as follows: 2 overhangs for *DrdI* and 0 overhangs for *BglI*. The number of sites with the 3' overhang having the same last 2 bases within the BAC clone used exactly once—singlets (i.e. @) is as follows: 3 of such *DrdI* sites and 1 of such *BglI* sites. The number of sites with the 3' overhang having the same last 2 bases within the BAC clone used exactly twice—doublets (i.e. #) is as follows: 1 of such *DrdI* sites and 5 of such *BglI* sites. The number of sites with the 3' overhang having the same last 2 bases within the BAC clone used more than twice (i.e. X) is as follows: 1 of such *DrdI* sites and 7 of such *BglI* sites.

Figures 13 shows the *SapI* site frequencies per 40kb in the Pendrin gene BAC from the 7q31 chromosome with the locations of the 14 *SapI* sites in a 97,943 bp clone being shown in pictorial and tabular form, indicating the type of overhang and the complement to that overhang. The number of sites with the 3' overhang having the same last 2 bases within the BAC clone used exactly once—singlets (i.e. @) is 7 of such *SapI* sites. The number of sites with the 3' overhang having the same last 2 bases within the BAC clone used exactly twice—doublets (i.e. #) is 2 of such *SapI* sites. The number of sites with the 3' overhang having the same last 2 bases within the BAC clone used more than twice (i.e. X) is 1 of such *SapI* sites.

Figure 14 shows the *DrdI* and *BglI* site frequencies per 40kb in the alpha2(I) collagen BAC from the 7q31 chromosome with the locations of the 11 *DrdI* and 15 *BglI* sites in a 116,466 bp clone being in pictorial and tabular form, indicating the type of overhang and the complement to that overhang. For this clone, per 40 kb, the unique sites are as follows: 1.4 of such unique *DrdI* sites and 3.1 of such unique *BglI* sites. In this clone, per 40 kb, the sites with the 3' overhang having the same last 2 bases--doublets (i.e. *) are as follows: 2.1 of such *DrdI* sites and 7.2 of such *BglI* sites. The number of palindromic overhangs not used (i.e. ^) is as follows: 1

overhang for *Drd*I and 0 overhangs for *Bgl*I. The number of sites with the 3' overhang having the same last 2 bases within the BAC clone used exactly once—singlets (i.e. @) is as follows: 2 of such *Drd*I sites and 4 of such *Bgl*I sites. The number of sites with the 3' overhang having the same last 2 bases within the BAC clone used exactly twice—doublets (i.e. #) is as follows: 4 of such *Drd*I sites and 7 of such *Bgl*I sites. The number of sites with the 3' overhang having the same last 2 bases within the BAC clone used more than twice (i.e. X) is as follows: 0 of such *Drd*I sites and 3 of such *Bgl*I sites.

Figures 15 shows the *Sap*I site frequencies per 40kb in the alpha2(I) collagen BAC from the 7q31 chromosome with the locations of the 18 *Sap*I sites in a 116,466 bp clone being in pictorial and tabular form, indicating the 18 *Sap*I site locations, the type of overhang, and the complement to that overhang. The number of sites with the 3' overhang having the same last 2 bases within the BAC clone used exactly once—singlets (i.e. @) is 4 of such *Sap*I sites. The number of sites with the 3' overhang having the same last 2 bases within the BAC clone used exactly twice—doublets (i.e. #) is 3 of such *Sap*I sites. The number of sites with the 3' overhang having the same last 2 bases within the BAC clone used more than twice (i.e. X) is 2 of such *Sap*I sites.

Figure 16 is a schematic drawing showing the sequencing of *Bgl*I islands in random BAC clones in accordance with the present invention.

Figure 16A is a schematic drawing showing the sequencing of *Bgl*I islands in random BAC clones using PCR amplification.

Figure 17 is a schematic drawing showing the sequencing of *Sap*I islands in random BAC clones in accordance with the present invention.

Figure 17A shows the probabilities of two or more singlets or doublets of *Drd*I, *Sap*I, or *Bgl*I sites in BAC clones containing 2 to 36 sites.

Figure 18 shows the alignment of BAC clone sequences, which are concordant and discordant, from *Drd*I sites.

Figure 19 shows *Drd*I/*Mse*I fragments in approximately 2 MB of human DNA. The average fragment size is about 125 bp, with most fragments being under 600 bp.

Figure 20 shows *DrdI*/*MspI*/*Taq*I fragments in approximately 2 MB of human DNA. The average fragment size is about 1,000 bp, with most fragments being over 600 bp.

Figure 21 shows how 4 unique singlet *Drd*I sequences are determined from 2 overlapping doublet BAC clone sequences.

Figure 22 shows how 3 unique singlet *Drd*I sequences are determined from overlapping doublet and triplet BAC clone sequences.

Figure 23 shows the *Bgl*I, *Drd*I, and *Sap*I sites in the pBeloBAC11 cloning vector.

Figure 24 shows the *Bgl*I, *Drd*I, and *Sap*I sites in the pUC19 cloning vector.

Figure 25 is a schematic drawing showing the sequencing of *Bam*HI islands in random BAC clones.

Figure 26 shows the *Eco*RI, *Hind*III, and *Bam*III site frequencies for the Met Oncogene in a sequenced BAC clone from the 7q31 chromosome. There are 19 *Bam*HI sites, 49 *Eco*RI sites, and the 64 *Hind*III sites within 171,905 bp clone as shown. The number of *Bam*HI sites that are the same where the 2 bases next to the site within the BAC clone are used exactly once—a singlet (i.e. @) is 6. The number of *Bam*HI sites that are the same where the 2 bases next to the site within the BAC clone are used exactly twice—a doublet (i.e. #) is 2. The number of *Bam*HI sites that are the same where the 2 bases next to the site within the BAC clone are used more than once is 2.

Figure 27 shows the *Avr*II, *Nhe*I, and *Spe*I site frequencies for the Met Oncogene in a sequenced BAC clone from the 7q31 chromosome. There are the 25 *Avr*II sites, 22 *Nhe*I sites, and the 21 *Spe*I sites within the 171,905 bp clone shown. The number of *Avr*II sites that are the same where the 2 bases next to the site within the BAC clone are used exactly once—a singlet (i.e. @) is 5. The number of *Avr*II sites that are the same where the 2 bases next to the site within the BAC clone are used exactly twice—a doublet (i.e. #) is 2. The number of *Avr*II sites that are the same where the 2 bases next to the site within the BAC clone are used more than once is 3. The number of *Nhe*I sites that are the same where the 2 bases next to the site within the BAC clone are used exactly once—a singlet (i.e. @) is 3. The number of

*Nhe*I sites that are the same where the 2 bases next to the site within the BAC clone are used exactly twice—a doublet (i.e. #) is 3. The number of *Nhe*I sites that are the same where the 2 bases next to the site within the BAC clone are used more than once is 3. The number of *Spe*I sites that are the same where the 2 bases next to the site within the BAC clone are used exactly once—a singlet (i.e. @) is 3. The number of *Avr*II sites that are the same where the 2 bases next to the site within the BAC clone are used exactly twice—a doublet (i.e. #) is 3. The number of *Avr*II sites that are the same where the 2 bases next to the site within the BAC clone are used more than once is 3.

Figure 28 is a schematic drawing showing the sequencing of *Bsi*HKAI islands in random BAC clones.

Figures 29 shows the *Acc*I and *Bsi*HKAI site frequencies for the Met Oncogene in a sequenced BAC clone from the 7q31 chromosome. 71 *Acc*I sites and 127 *Bsi*HKAI sites within 171,905 bp clone are shown. The number of *Acc*I sites that are the same where the 2 bases next to the site within the BAC clone are used exactly once—a singlet (i.e. @) is 4. The number of *Acc*I sites that are the same where the 2 bases next to the site within the BAC clone are used exactly twice—a doublet (i.e. #) is 2. The number of *Acc*I sites that are the same where the 2 bases next to the site within the BAC clone are used more than once is 0. The number of *Bsi*HKAI sites that are the same where the 2 bases next to the site within the BAC clone are used exactly once—a singlet (i.e. @) is 6. The number of *Bsi*HKAI sites that are the same where the 2 bases next to the site within the BAC clone are used exactly twice—a doublet (i.e. #) is 3. The number of *Bsi*HKAI sites that are the same where the 2 bases next to the site within the BAC clone are used more than twice is 0.

Figure 30 is a schematic drawing showing the sequencing of *San*DI islands in random BAC clones.

Figure 31 shows the *San*DI and *Sex*AI site frequencies for the Met Oncogene in a sequenced BAC clone from the 7q31 chromosome. There are 13 *San*DI sites and 15 *Sex*AI within the 171,905 bp clone. The number of *San*DI sites that are the same where the 2 bases next to the site within the BAC clone are used exactly once—a singlet (i.e. @) is 3. The number of *San*DI sites that are the same where the 2 bases next to the site within the BAC clone are used exactly twice—a

doublet (i.e. #) is 5. The number of *San*DI sites that are the same where the 2 bases next to the site within the BAC clone are used more than once is 0. The number of *Sex*AI sites that are the same where the 2 bases next to the site within the BAC clone are used exactly once—a singlet (i.e. @) is 8. The number of *Sex*AI sites that are the same where the 2 bases next to the site within the BAC clone are used exactly twice—a doublet (i.e. #) is 2. The number of *Sex*AI sites that are the same where the 2 bases next to the site within the BAC clone are used more than twice is 1.

Figure 32 shows the *Acc*I and *Bsi*HKAI sites in the pBeloBAC11 cloning vector. There are 6 *Acc*I sites and 8 *Bsi*HKAI sites.

Figure 33 shows the *Avr*II, *Bam*HI, *Nhe*I, and *Spe*I sites in the pBeloBAC11 cloning vector.

Figures 34 shows the *San*DI and *Sex*AI sites in the pBeloBAC11 cloning vector.

Figure 35 shows the *Drd*I, *Bgl*I, *Sap*I, *Taq*I, and *Msp*I sites in a sequenced BAC cloning vector from the 7q31chromosome. There are 12 *Drd*I sites, 16 *Bgl*I sites, 25 *Sap*I sites, 63 *Taq*I sites, and 86 *Msp*I sites in the 171,905 base pairs..

Figure 36 shows the three degrees of specificity in amplifying a *Bgl*I representation.

Figure 37 shows Scheme 1 for sequencing for *Drd*I and *Bgl*I representations of individual BAC clones.

Figure 38 shows overlapping *Drd*I islands in four hypothetical BAC clones using AA overhangs.

Figure 39 shows overlapping *Drd*I islands in four hypothetical BAC clones using AC overhangs.

Figure 40 shows overlapping *Drd*I islands in four hypothetical BAC clones using AG overhangs.

Figure 41 shows overlapping *Drd*I islands in four hypothetical BAC clones using CA overhangs.

Figure 42 shows overlapping *Drd*I islands in four hypothetical BAC clones using GA overhangs.

Figure 43 shows overlapping *Drd*I islands in four hypothetical BAC clones using GG overhangs.

Figure 44 shows overlapping *Drd*I islands in four hypothetical BAC clones using AA, AC, AG, CA, GA, and GG overhangs.

Figure 45 shows the alignment of the four hypothetical BAC clones based upon on the unique and overlapping *Drd*I islands depicted in Figures 38 to 44.

Figure 46 shows the sizes of representational fragments generated by *Drd*I, *Taq*I and *Msp*I digestion in overlapping BACs from 7q31. When such fragments are amplified using linker ligation/PCR amplification, they will contain approximately 25 additional bases on each side. Sizes of fragments were determined from 3 separate contigs on 7q31 known as contig 1941 (BACs RG253B13, RG013N12, and RG300C03), contig T002144 (BACs RG022J17, RG067E13, RG011J21, RG022C01, and RG043K06), and contig T002149 (RG343P13, RG205G13, O68P20, and H_133K23). Overlaps between BACs in contig 1941 are indicated by the following symbols: RG253B13/ RG013N12 = *, RG013N12/R RG300C03 = †. Overlaps between BACs in contig T002144 are indicated by the following symbols: RG022J17/RG067E13 = *, RG067E13/RG011J21 = †, RG011J21 / RG022C01 = ‡, and RG022C01/ RG043K06 = **. Overlaps between BACs in contig T002149 are indicated by the following symbols: RG343P13/ RG205G13 = *, RG205G13/ O68P20 = †, and O68P20/ H_133K23 = ‡.

Figure 47 shows the sizes of representational fragments generated by *Drd*I and *Mse*I digestion in overlapping BACs from 7q31. When such fragments are amplified using linker ligation/PCR amplification, they will contain approximately 25 additional bases on each side. Sizes of fragments were determined from 3 separate contigs on 7q31 known as contig 1941 (BACs RG253B13, RG013N12, and RG300C03), contig T002144 (BACs RG022J17, RG067E13, RG011J21, RG022C01, and RG043K06), and contig T002149 (RG343P13, RG205G13, O68P20, and H_133K23). Overlaps between BACs in contig 1941 are indicated by the following symbols: RG253B13/ RG013N12 = *, RG013N12/R RG300C03 = †. Overlaps between BACs in contig T002144 are indicated by the following symbols: RG022J17/RG067E13 = *, RG067E13/RG011J21 = †, RG011J21 / RG022C01 = ‡, and RG022C01/ RG043K06 = **. Overlaps between BACs in contig T002149 are indicated by the following symbols: RG343P13/ RG205G13 = *, RG205G13/ O68P20 = †, and O68P20/ H_133K23 = ‡.

Figure 48 shows the *DrdI*, *TaqI*, and *MspI* sites in 4 sequenced BAC clones from a 7q31c chromosome as well as the location and identities of the AA, AC, AG, CA, GA, and GG overhangs and their overhangs.

Figure 49 is a schematic drawing showing the PCR amplification of a *DrdI* representation for shotgun cloning and generating mapped SNPs.

Figure 49A is a schematic drawing of the PCR amplification of a *DrdI* representation for shotgun cloning and generating mapped SNPs.

Figure 50 is a schematic drawing showing the PCR amplification of a *DrdI* representation for high-throughput SNP detection.

Figure 50A is an alternative schematic drawing showing the PCR amplification of a *DrdI* representation for high-throughput SNP detection.

Figures 51A-B show the quantitative detection of G12V mutation of the K-*ras* gene using two LDR probes in the presence of 10 micrograms of salmon sperm DNA. Figure 51A is a graph showing the amount of LDR product formed is a linear function of K-*ras* mutant DNA template, even at very low amounts of template. Figure 51B is a log-log graph of amount of LDR product formed for various amount of K-*ras* mutant DNA in a 20 µl LDR reaction. The amount of LDR product formed with 2.5 pM (50 amol) to 3 nM (60 fmol) of mutant K-*ras* template was determined in duplicate using fluorescent probes on an ABD 373 DNA sequencer.

Figures 52A-B show a scheme for PCR/LDR detection of mutations in codons 12 and 13 of K-*ras*. using an addressable array. Figure 52A shows a schematic representation of chromosomal DNA containing the K-*ras* gene. Exons are shaded and the position of codons 12 and 13 are shown. Exon-specific primers were used to selectively amplify K-*ras* DNA flanking codons 12 and 13. Probes were designed for LDR detection of seven possible mutations in these two codons. Discriminating LDR probes contained a complement to an address sequence on the 5' end and the discriminating base on the 3' end. Common LDR probes were phosphorylated on the 5' end and contained a fluorescent label on the 3' end. Figure 52B shows the presence and type of mutation is determined by hybridizing the contents of an LDR reaction to an addressable DNA array. The capture oligonucleotides on the array have sequences which are designed to be sufficiently different, so that only probes containing the correct complement to a given capture

oligonucleotide remain bound at that address. In the LDR reaction, only a portion of the hybrid probe is ligated to its adjacent common fluorescently labeled probe (in the presence of the correct target). Thus, for every hybridization, an identical quantity of addressable array-specific portion competes for hybridization to each address. This feature allows for simultaneous identification and quantification of LDR signal.

Figure 53 shows the array hybridization of K-*ras* LDR products. Arrays were hybridized for 1 hour at 65 °C in a hybridization oven with nine individual LDR reactions (17 µL) diluted to 55 µL with 1.4X hybridization buffer. Following hybridization, arrays were washed for 10 minutes at room temperature in 300 mM bicine pH 8.0, 10 mM $MgCl_2$, 0.1% SDS. The arrays were analyzed on an Olympus AX70 epifluorescence microscope equipped with a Princeton Instruments TE/CCD-512 TKBM1 camera. The images were collected using a 2 second exposure time. All nine arrays displayed signals corresponding to the correct mutant and/or wild-type for each tumor or cell line sample. The small spots seen in some of the panels, i.e. near the center of the panel containing the G13D mutant, are not incorrect hybridizations, but noise due to small bubbles in the polymer.

Figures 54A-B show the quantification of minority fluorescently-labeled oligonucleotide probe captured by a universal addressable array using two different detection instruments. Hybridizations were carried out using 55 µl hybridization buffer containing 4,500 fmole fluorescently-labeled common probes, 9 x 500 fmole of each unlabeled, addressable array-specific portion-containing discriminating probe, and 1 to 30 fmol CZip13 oligonucleotide. Figure 54A shows the quantification of the amount of captured CZip13 oligonucleotide using a Molecular Dynamics 595 FluorImager. Figure 54B shows the quantification of the amount of captured CZip13 oligonucleotide using an Olympus AX70 epifluorescence microscope equipped with a Princeton Instruments TE/CCD-512 TKBM1 camera.

Figure 55 shows how an allelic imbalance can be used to distinguish gene amplification from loss of heterozygosity (i.e. LOH) in tumor samples which contain stromal contamination.

Figure 56 shows the PCR/LDR quantification of different ratios of K-*ras* G12V mutant to wild-type DNA. LDR reactions were carried out in a 20 µl

reaction containing 2 pmol each of the discriminating and wild type ("wt") probe, 4 pmol of the common probe and 1 pmol total of various ratios of PCR product (pure wt and pure G12V mutant) from cell lines (HT29 and SW620). LDR reactions were thermally cycled for 5 cycles of 30 sec at 94°C and 4 min. at 65°C, and quenched on ice. 3 µl of the LDR reaction product was mixed with 1 µl of loading buffer (83% formamide, 83 mM EDTA, and 0.17% Blue Dextran) and 0.5 ml TAMRA 350 molecular weight marker, denatured at 94°C for 2 minutes, chilled rapidly on ice prior to loading on a 8 M urea-10% polyacrylamide gel, and electrophoresed on an ABI 373 DNA sequencer at 1400 volts. Fluorescent ligation products were analyzed and quantified using the ABI GeneScan 672 software (Perkin-Elmer Biosystems, Foster City, CA). The amount of product obtained was calculated using the peak area and from the calibration curve (1 fmol = 600 peak area units). The normalized ratio was obtained by multiplying or dividing the absolute ratio by the 1:1 absolute ratio.

Figures 57A-B are schematic drawings showing PCR/LDR procedures using addressable DNA arrays where there are 2 alternative labeling schemes for capture on the array.

Figure 58 is a schematic diagram showing a labeling scheme for PCR/SNUPE with addressable array capture.

Figure 59 is a diagram showing a labeling scheme for PCR/LDR with gene array capture.

Figure 60 is a schematic diagram showing a labeling scheme for LDR/PCR with addressable array capture.

Figure 61 is a diagram showing a labeling scheme for LDR/PCR with lambda exonuclease digestion and addressable array capture.

Figures 62A-B are schematic drawings showing 2 alternative dual label strategies to quantify LDR signal using addressable DNA arrays.

Figure 63 shows the detection of gene amplification in tumor samples which contain stromal contamination using addressable array-specific portions on the discriminating oligonucleotide probe.

Figure 64 shows the detection of gene amplification in tumor samples which contain stromal contamination using addressable array-specific portions on the common oligonucleotide probe.

Figure 65 shows the detection of heterozygosity (i.e. LOH) in tumor samples which contain stromal contamination using addressable array-specific portions on the discriminating oligonucleotide probes.

Figure 66 shows the detection of heterozygosity (i.e. LOH) in tumor samples which contain stromal contamination using addressable array-specific portions on the common oligonucleotide probes.

Figure 67 shows the calculations for the detection procedure shown in Figure 63.

Figure 68 shows the calculations for the detection procedure shown in Figure 64.

Figure 69 shows the calculations for the detection procedure shown in Figure 65.

Figure 70 shows the calculations for the detection procedure shown in Figure 66.

Figure 71 shows the fidelity of T4 DNA ligase on synthetic target/linker. T4 DNA ligase assays were performed with linkers containing 2 base 3' overhangs (GG, AA, AG, and GA) and synthetic targets containing 2 base 3' complementary or mismatched overhangs (CC, TT, TC, and CT). Products represent both top and bottom strand ligation products. Synthetic targets were designed such that the common strand (top strand) provided a 39 nucleotide product (common product), while the specific strand (bottom strand) provided a 48 (CC, TT), 52 (CT), or 56 (TC) nucleotide product. Only the correct complement product is observed, while there were no misligations. Since TT- and CC- targets result in the same length products, TT-targets are not present in GG-linker assays and CC-targets are not present in AA-linker assays. For AG- and GA-linker assays, all four targets (TC-, CT-, CC-, and TT-) are present. Synthetic complementary target was present at 5 nM, and each linker/adapter was present at either 50 nM (=10x concentration), or 500 nM (=100x concentration).

Figure 72 shows *DrdI* representations of human genomic DNA. The *DrdI* representation of human genomic DNA was generated by "regular PCR" and "touchdown PCR" using 3 and 4 base selection PCR primers. The six lanes following the 100 bp ladder lane were the PCR amplification of *DrdI* AG- overhang fragments

of human genome by regular PCR and touchdown PCR using AGC, AGA, AGAT, and AGAG selection primers, respectively. The last six lanes were the PCR amplification of *Drd*I CA- overhang fragments of human genome by regular PCR and touchdown PCR using CAG, CAT, CAGT, and CATG selection primers, respectively.

Figure 73 shows the sensitivity of a PCR/LDR reaction. Human genomic DNA was subjected to PCR amplification using region specific primers, followed by LDR detection using LDR probes specific to the amplified regions. Aliquots of 3 μl of the reaction products were mixed with 3 μl of loading buffer (83% formamide, 8.3 mM EDTA, and 0.17% Blue Dextran) and 0.5 μl Rox-1000, or TAMRA 350 molecular weight marker, denatured at 94°C for 2 min., chilled rapidly on ice prior to loading on an 8 M urea-10% polyacrylamide gel, and electrophoresed on an ABI 373 DNA sequencer at 1400 volts. Fluorescent ligation products were analyzed and quantified using the ABI Gene Scan software. The first six lanes were the results of an LDR assay of PCR amplified human genomic DNA using probes which amplify fragments which should be present in AGA *Drd*I representations; without salmon sperm DNA, and 500, 1,500, 4,500, 13,500 fold dilutions in 10 μg salmon sperm DNA, and 10 μg salmon sperm DNA alone, respectively. The last six lanes were the results of an LDR assay of PCR amplified human genomic DNA using probes which amplify fragments which should be present in AGC *Drd*I representations; without salmon sperm DNA, and 500, 1,500, 4,500, 13,500 fold dilutions in 10 μg salmon sperm DNA, and 10 μg salmon sperm DNA alone, respectively.

Figure 74 shows LDR detection of AG- overhang representations of the human genome. *Drd*I representations were generated by the "regular PCR" and the "touchdown PCR" using common probe MTCG228 and 3 and 4 base selection PCR primers AGAP60, AGCP61, AGATP62, and AGAGP63. The presence of specific fragments in the representation were detected by LDR using probes specific to the amplified regions (Tables 16). In the REF lane, used as the standard, were LDR results of PCR products generated from probes designed for each of the targeted regions in the human genome. The labels on the left refer to the four bases present at the *Drd*I site and the number in parenthesis represents the predicted length of the

*Drd*I-*Msp*I/*Taq*I fragment. The four lanes following the REF lane were the LDR results of detecting representation generated by regular PCR and touchdown PCR using AGC reach in primer AGCP61, respectively. The four lanes under AGA representation were the LDR results of detecting representation generated by regular PCR and touchdown PCR with AGA reach in primer AGAP60, respectively. The four lanes under AGAT representation were the LDR results of detecting representation generated by regular PCR and touchdown PCR with AGAT reach in primer AGATP62, respectively. The four lanes under AGAG representation were the LDR results of detecting representation generated by regular PCR and touchdown PCR with AGAG reach in primer AGAGP63, respectively.

Figure 75 shows LDR detection of CA- overhang representations of the human genome. *Drd*I representations were generated by the "regular PCR" and the "touchdown PCR" using common probe MTCG228 and 3 and 4 base selection PCR primers CATP58, CAGP59, CATGP64, and CAGTP65. Presence of specific fragments in the representation were detected by LDR using probes specific to the amplified regions (Table 17). In the REF lane, used as the standard, were LDR results of PCR products generated from probes designed for each of the targeted regions in the human genome. The labels on the left refer to the four bases present at the *Drd*I site and the number in parenthesis represents the predicted length of the *Drd*I-*Msp*I/*Taq*I fragment. The four lanes following REF lane were the LDR results of detecting representations generated by "regular PCR" with CAGP59, CATP58, CAGTP65, and CATGP64 reach in probes, respectively. The last four lanes were the LDR results of detecting representations generated by "touchdown PCR" with CAGP59, CATP58, CAGTP65, and CATGP64 reach in probes, respectively.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is directed to a method of assembling genomic maps of an organism's DNA or portions thereof. A library of an organism's DNA is provided where the individual genomic segments or sequences are found on more than one clone in the library. Representations of the genome are created, and nucleic acid sequence information is generated from the representations. The sequence

information is analyzed to determine clone overlap from a representation. The clone overlap and sequence information from different representations is combined to assemble a genomic map of the organism.

Summary of *Drd*I island approach to accelerate alignment of clones.

The *Drd*I island approach obtains a representation of the sequence in a genome which may be used to complete the map of the genome, to find mapped SNPs, and to evaluate genome differences and their association with diseases.

The first step of the procedure is to form a library of genomic DNA in cosmid, bacteriophage P1, or bacterial artifical chromosome ("BAC") clones. Each clone of the library is cut with a restriction enzyme into a plurality of fragments which have degenerate ends. Unique linkers are ligated to the degenerate ends. Internal sequence information in the clones may be obtained by sequencing off the linkers. This creates 1kb "islands" of sequence surrounding the restriction sites which are within that clone. In essence, a "representation" of the genome in the form of "islands" is created, but the islands are attached to random clones and hence the clone overlap can be determined.

Depending on the particular restriction site used, an average of 5-8 different sets of sequencing runs are performed on the random clones (and up to 16 if needed), creating the representations of the genome. The sequence information from one set (e.g., a sequencing primer ending with 3' AA) may be used to align clones based on an analysis of overlaps between singlet, doublet, and even triplet reads. In addition, a given clone contains interpretable sequence information from at least two sets, and often from all 5-8 sets. Thus, the information from different sets on the same clone may also be used to align clones.

Once an overlapping map of the human genome is created, it becomes a powerful tool for completing the entire genomic sequence as well as identifying mapped SNPs. This procedure permits 100,000 SNPs to be identified by a shotgun method which immediately gives their map position. Further, these SNPs are amenable for use in a high throughput detection scheme which uses a universal DNA array.

I. Preparation of Genomic DNA

In order to carry out the mapping procedure of the present invention, the genomic DNA to be mapped needs to be divided into a genomic library comprising a plurality of random clones. The genomic library can be formed and inserted into cosmid clones, bacteriophage P1 vectors, or bacterial artificial chromosome clones ("BAC") as described in Chapters 2, 3, and 4 of Birren, et. al., Genomic Analysis—A Laboratory Manual Vol. 3 (Cold Spring Harbor Laboratory Press 1997), which is hereby incorporated by reference.

When producing cosmid clones, a genomic DNA library may be constructed by subjecting a sample of genomic DNA to proteinase K digestion followed by partial enzymatic digestion with *Mbo*I to form DNA fragments of random and varying size of 30-50kb. Cosmid vectors with single *cos* sites can be digested with *Bam*HI to linearize the vector followed by dephosphorylation to prevent religation. Cosmid vectors with dual *cos* sites can be digested with *Xba*I to separate the two cosmid sites and then dephosphorylated to prevent religation. The vector and genomic DNA are ligated and packaged into lambda phage heads using *in vitro* packaging extract prepared from bacteriophage lambda. The resulting phage particles are used to infect an *E. coli* host strain, and circularization of cosmid DNA takes place in the host cell.

In forming bacteriophage P1 vector libraries, genomic DNA is subjected to partial digestion with a restriction enzyme like *Sau*3AI followed by size fractionation to produce 70 to 100 kb DNA fragments with *Sau*3AI 5' overhangs at each end. A bacteriophage P1 cloning vector can be treated sequentially with the *Sca*I and *Bam*HI restriction enzymes to form short and long vector arms and dephosphorylated with BAP or CIP to prevent religation. The *pac* site can then be cleaved by incubation with an extract prepared by induction of a bacteriophage lysogen that produces appropriate bacteriophage P1 *pac* site cleavage proteins (i.e. Stage I reaction). After the *pac* site is cleaved, the DNA is incubated with a second extract prepared by induction of a bacteriophage lysogen that synthesizes bacteriophage P1 heads and tails but not *pac* site cleavage proteins (i.e. Stage II

reaction). The genomic DNA and vector DNA are then ligated together followed by treatment with Stage I and, then, Stage II extract of *pac* site cleavage proteins. Unidirectional packaging into the phage head is initiated from the cleaved *pac* end. After the phage head is filled with DNA, the filled head is joined with a phage tail to form mature bacteriophage particles. The P1 DNA is then incorporated into a bacterial host cell constitutively expressing the *Cre* recombinase. The phage DNA is cyclized at *lox*P sites, and the resulting closed circular DNA is amplified.

In producing BAC libraries, genomic DNA in agarase is subjected to partial digestion with a restriction enzyme followed by size separation. BAC vectors are digested with a restriction enzyme and then dephosphorylated to prevent religation. Suitable restriction enzymes for digestion of the BAC vectors include *Hin*dIII, *Bam*HI, *Eco*RI, and *Sph*I. After conducting test ligations to verify that clones with low background will be produced, the genomic DNA and BAC DNA are ligated together. The ligated genomic and BAC DNA is then transformed into host cells by electroporation. The resulting clones are plated.

II. *Drd*I Island Approach

A Single Restriction/Ligation Reaction is Used to Obtain Internal Sequences of Clones at *Drd*I Sites.

Once the individual clones are produced from genomic DNA and separated from one another, as described above, the individual clones are treated in accordance with the *Drd*I approach of the present invention.

Figure 1 is a schematic drawing showing the sequencing of *Drd*I islands in random plasmid or cosmid clones in accordance with the present invention. The random plasmid or cosmid clones produced as described above are amplified. Nucleic acid amplification may be accomplished using the polymerase chain reaction process. The polymerase chain reaction process is the preferred amplification procedure and is fully described in H. Erlich, et. al., "Recent Advances in the Polymerase Chain Reaction," Science 252: 1643-50 (1991); M. Innis, et. al., PCR Protocols: A Guide to Methods and Applications, Academic Press: New York (1990); and R. Saiki, et. al., "Primer-directed Enzymatic Amplification of DNA with

a Thermostable DNA Polymerase," Science 239: 487-91 (1988), which are hereby incorporated by reference. Long range PCR procedures are described in Cheng, et al., "Long PCR," Nature, 369(6482):684-5 (1994) and Cheng, et al., "Effective Amplification of Long Targets From Cloned Inserts and Human Genomic DNA," Proc Natl Acad Sci USA, 91(12): 5695-9 (1994), which are hereby incorporated by reference.

In carrying out a polymerase chain reaction process, the target nucleic acid, when present in the form of a double stranded DNA molecule, is denatured to separate the strands. This is achieved by heating to a temperature of 85-105°C. Polymerase chain reaction primers are then added and allowed to hybridize to the strands, typically at a temperature of 50-85°C. A thermostable polymerase (e.g., *Thermus aquaticus* polymerase) is also added, and the temperature is then adjusted to 50-85°C to extend the primer along the length of the nucleic acid to which the primer is hybridized. After the extension phase of the polymerase chain reaction, the resulting double stranded molecule is heated to a temperature of 85-105°C to denature the molecule and to separate the strands. These hybridization, extension, and denaturation steps may be repeated a number of times to amplify the target to an appropriate level.

The amplified clones are then incubated with a *Drd*I restriction enzyme, a T4 ligase, and a linker at 15°C to 42°C, preferably 37°C, for 15 minutes to 4 hours, preferably 1 hour. As shown in Figure 1, the *Drd*I restriction enzyme cuts both strands of the clone where indicated by the arrows and the T4 ligase couples a doubled stranded linker to the right hand portion of the cut clone to form a double stranded ligation product, as shown in Figure 1. In the embodiment depicted, the linker has an AA overhang, but, as discussed *infra*, *Drd*I will cut any 6 bases between a GAC triplet and GTC triplet, leaving a 3' double base (i.e. NN) overhang. Therefore, the *Drd*I island technique of the present invention utilizes a different linker for each of the non-palindromic, 3' double base overhangs to be identified.

After the different linkers are ligated to the fragments of DNA produced by *Drd*I digestion to form a phosphorylated site containing, in the case of Figure 1, a 3' AA overhang, the T4 ligase and the restriction enzyme (i.e. *Drd*I) are

inactivated by heating at 65°C to 98°C, preferably 95°C, for 2 minutes to 20 minutes, preferably 5 minutes. As shown in Figure 1, a sequencing primer is contacted with the ligation product after it is denatured to separate its two strands. For the linker depicted, the sequencing primer has a 3' AA overhang and nucleotides 5' to the overhang which makes the primer suitable for hybridization to one strand of the ligation product. Sequencing primers adapted to hybridize to the ligation products formed from the other linkers are similarly provided. With such sequencing primers, a dideoxy sequencing reaction can be carried out to identify the different *Drd*I cleavage sites. Dideoxy sequencing is described in Chadwick, et al., "Heterozygote and Mutation Detection by Direct Automated Fluorescent DNA Sequencing Using a Mutant Taq DNA Polymerase," Biotechniques, 20(4):676-83 (1996) and Voss, et al., "Automated Cycle Sequencing with Taquenase: Protocols for Internal Labeling, Dye Primer and 'Doublex' Simultaneous Sequencing," Biotechniques, 23(2):312-8 (1997), which are hereby incorporated by reference. In situations where the results of dideoxy sequencing with primers having a 2 base 3' end (i.e. NN) are too difficult to interpret due to priming three or more fragments during the sequencing reaction, additional selectivity can be achieved by performing 4 separate dideoxy sequencing reactions for each linker. For example, with respect to the linker 3' AA overhang, sequencing primers having 3' ends of AAA, AAC, AAG, and AAT can be utilized to obtain sequences for *Drd*I cleavages filled with the AA-containing linker. This technique is amenable to automation. In cases where there is insufficient DNA template to conduct dideoxy sequencing, this sequencing step can be preceded by a PCR amplification procedure. Suitable PCR amplification conditions are described above.

The results of the above-described sequencing procedure indicates the number of times a particular linker sequence is present in an individual clone. If a particular linker sequence appears only one time in a given clone, it is referred to as a unique or singlet sequence, while the presence of a particular linker sequence two times is referred to a doublet, three times is referred to a triplet, etc. The fragments with the different 2 base overhangs (e.g., AA, AC, AG, CA, GA, and GG) constitute representations, and the representations for different clones are then examined to

determine if there is any commonality (i.e. the clones overlap). Based on this analysis, the different clones are assembled into a genomic map.

The enzyme *DrdI* (GACNNNN^NNGTC leaves a 3' NN overhang in the middle of 6 bases of degenerate sequence. The 16 NN sites which may be created fall into three groups -- self-complementary (Group I), 6 non-complementary (Group II), and the other 6 non-complementary dinucleotides (Group III) as follows.

Group I	Group II	Group III
CG	AG	CT
GC	AC	GT
AT	CA	TG
TA	GA	TC
	AA	TT
	GG	CC

Group I has complementary overhangs. Thus, a given linker would ligate to both sides of the cut site, so sequencing reactions would provide double reads on the same lane and would not be worth pursuing. Further, the complementary linkers can ligate to each other, forming primer dimers. Therefore, sites which generate CG, GC, AT, or TA ends will be ignored.

Groups II and III are ideal. Linkers with unique sequences (for a subsequent sequencing run) ending in AG, AC, CA, GA, AA, and GG can be used in a first ligation reaction. Linkers ending in the other six dinucleotides (i.e. CT, GT, TG, TC, TT, and CC) can be used in a second ligation reaction.

To reduce the number of sequencing runs needed, sequences should be obtained from the overhang which requires linker adapters whose 3' two bases end in AA, AC, AG, CA, GA, and GG. This avoids use of both linkers and sequencing primers which contain or end in a "T" base. Such linkers or primers are more susceptible to misligations or mispriming since T-G mismatches give higher rates of misligation or polymerase extension than any of the other mismatches.

The advantage of using *DrdI* is that it leaves a 2 base 3' overhang on a split palindrome. Thus, the product of a PCR reaction may be immediately used in a

*Drd*I restriction/ligation reaction, without requiring time consuming purification of the PCR fragment. Polymerase won't extend the 3' overhang ends generated by *Drd*I.

*Drd*I sites are eliminated by ligation of the linkers, but are recreated and cut again if two PCR fragments are ligated together. The *Drd*I linker is phosphorylated so both strands ligate. Since the end is non-complementary, it cannot ligate to itself. Thus, all free *Drd*I ends will contain linkers.

As noted above, the linkers of Group II or Group III can used together. As shown in Figures 2 and 3, there are 2 schemes for separately carrying out each of the *Drd*I island sequencing procedures for each group.

As shown in Figure 2, one scheme invoives using a single tube or well: (1) to PCR amplify or partially purify DNA from individual clones from the cosmid, PAC, or BAC libraries; (2) to incubate with *Drd*I, T4 ligase, and the 6 divergent linkers with nonpalindromic 3' double base overhangs; and, optionally, (3) to PCR amplify to generate sufficient DNA template for dideoxy sequencing. At this point, the material to be sequenced is aliquoted into multiple (e.g., 6) tubes or wells with each tube or well being used to carry out one of the 6 separate sequencing reactions for each of the *Drd*I cleavage sites filled by the 6 linkers of Group II or Group III. If sequencing primers with an additional base are needed to overcome sequencing reads which are difficult to interpret (as discussed above), these primers can be added to the tube or well used to carry out the sequencing of the cleavage site for their respective linker.

Figure 2 provides a scheme for sequencing representations of BAC clones. Two approaches may be considered for preparing DNA. One rapid approach is to pick individual colonies into lysis buffer and lyse cells under conditions which fragment chromosomal DNA but leave BAC DNA intact. Chromosomal DNA is digested by the ATP dependent DNase from Epicentre which leaves CCC and OC BAC DNA intact. After heat treatment to inactivate the DNase, restriction digestion, ligation of linker adapters, and PCR amplification are all performed in a single tube. The products are then aliquoted and sequencing is performed using specific primers to the adapters. This first approach has the advantage of obviating the need to grow and store 300,000 BAC clones.

An alternative approach is to pick the colonies into 1.2 ml growth media and make a replica into fresh media for storage before pelleting and preparing crude BAC DNA from a given liquid culture similar as described above. This second approach has the advantage of producing more BAC DNA, such that loss of an island from PCR dropout is less likely. Further, this approach keeps a biological record of all the BACs, which may become useful in the future for techniques such as exon trapping, transfection into cells, or methods as yet undeveloped.

As shown in Figure 3, the second scheme involves using a single tube or well to PCR amplify or partially purify DNA from individual clones from the cosmid, PAC, or BAC libraries. The PCR product can then be aliquoted into multiple (e.g., 6) tubes or wells: (1) to incubate with *DrdI*, T4 ligase, and the 6 divergent linkers with nonpalindromic 3' double base overhangs; (3), optionally, to PCR amplify to generate sufficient DNA template for dideoxy sequencing; and (3) to carry out one of the 6 separate sequencing reactions for each of the *DrdI* cleavage sites filled by the 6 linkers of Group II or Group III. As to step (3), if sequencing primers with an additional base are needed to overcome sequencing reads which are difficult to interpret (as discussed above), these primers can be added to the tube or well used to carry out the sequencing of the cleavage site for their respective linker.

As shown in Figure 4, DNA sequencing can be carried out directly from PCR-amplified DNA without primer interference, the PCR primers from the original PCR reaction may be removed by using riboU containing primers and destroying them with either base or (using dU) with UNG. This is achieved by incorporating ribonucleotides directly into PCR primers. Colonies are then picked into microwell PCR plates. The primers containing ribose, on average every fourth nucleotide, are added. The preferred version would use r(U) in place of dT, which simplifies synthesis of primers. After PCR amplification, in the presence of dNTPs and *Taq* polymerase, 0.1N NaOH is added and the PCR product is heated at 95°C for 5 minutes to destroy unused primers. The PCR product is then diluted to 1/10th of the volume in 2 wells and forward and reverse sequencing primers are added to run fluorescent dideoxy sequencing reactions.

Another approach to sequence DNA directly from PCR-amplified DNA uses one phosphorylated primer, lambda exonuclease to render that strand and

the primer single stranded, and shrimp alkaline phosphatase to remove dNTPs. This is commercially available in kit form from Amersham Pharmacia Biotech, Piscataway, NJ. A more recent approach to sequence DNA directly from PCR-amplified DNA uses ultrafiltration in a 96 well format to simply remove primers and dNTPs physically, and is commercially available from Millipore, Danvers, MA.

Figure 5 shows an alternative embodiment of the *Drd*I island sequencing procedure of the present invention. In this embodiment, individual BAC clones are cut with the restriction enzymes *Drd*I and *Msp*I in the presence of linkers and T4 ligase. This is largely the same procedure as that described with reference to Figure 1 except that the *Msp*I restriction enzyme is utilized to reduce the length of the fragment to a size suitable for PCR amplification. In Figure 5, the subtleties of the linker-adapter ligations and bubble PCR amplification to select only the *Drd*I-*Msp*I fragments are detailed. As in Figure 1, the linker for the *Drd*I site is phosphorylated and contains a 3' two base overhang (e.g., a 3' AA overhang as in Figure 5). A separate linker is used for the *Msp*I site which replaces the portion of the BAC DNA to the right of the *Msp*I site in Figure 5. The *Msp*I linker is not phosphorylated and contains a bubble (i.e. a region where the nucleotides of this double stranded DNA molecule are not complementary) to prevent amplification of unwanted *Msp*I-*Msp*I fragments. The T4 ligase binds the *Drd*I and *Msp*I linkers to their respective sites on the BAC DNA fragments with biochemical selection assuring that most sites contain linkers.

After the different linkers are ligated to the fragments of DNA produced by *Drd*I digestion to form a phosphorylated site containing, in the case of Figure 5, a 3' AA overhang, the T4 ligase and the restriction enzymes (i.e. *Drd*I and *Msp*I) are inactivated by heating at 65°C to 98°C, preferably 95°C, for 2 minutes to 20 minutes, preferably 5 minutes. As shown in Figure 5, the ligation product is amplified using a PCR procedure under the conditions described above. For the linker depicted, one amplification primer has a 3' AA overhang and nucleotides 5' to the overhang which makes the primer suitable for hybridization to the bottom strand of the ligation product for polymerization in the 3' to 5' direction. The other sequencing primer, for the linker depicted in Figure 5, has a 5' CG overhang which makes this primer suitable for hybridization to the top strand of the ligation product

for polymerization in the 5' to 3' direction. Amplification primers adapted to hybridize to the ligation products formed from the other linkers are similarly provided. As described with reference to Figure 4, PCR amplification is carried out using primers with ribose U instead of dT, adding dNTPs and *Taq* polymerase, adding NaOH, and heating at 85°C to 98°C, preferably 95°C, for 2 minutes to 20 minutes, preferably 5 minutes to inactivate any unused primer.

After amplification is completed and the amplification product is neutralized and diluted, dideoxy sequencing can be conducted in substantially the same manner as discussed above with reference to Figure 1. If necessary, a separate dideoxy sequencing procedure can be conducted using a sequencing primer which anneals to the *Msp*I site linker. This is useful to generate additional sequence information associated with the *Drd*I island.

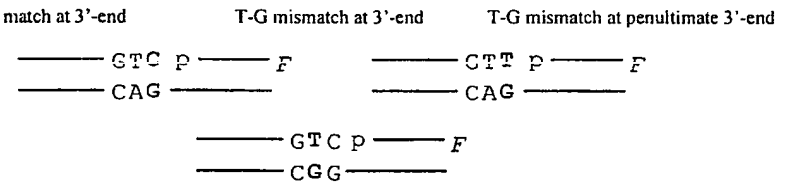
Figure 6 shows a variation of the scheme for amplifying *Drd*I islands for sequencing directly from small quantities of BAC DNA. Individual BAC clones are cut with the restriction enzymes *Drd*I, *Msp*I, and *Taq*I in the presence of linkers and T4 ligase. This is largely the same procedure as described in Figure 5 except that the *Msp*I and *Taq*I restriction enzymes are used to reduce the length of the fragment to a size suitable for PCR amplification. As in Figure 5, the linker for the *Drd*I site is phosphorylated and contains a 3' two base overhang (e.g., a 3' AA overhang as in Figure 6). A separate linker is used for the *Msp*I or *Taq*I site which replaces the portion of the BAC DNA to the right of the *Msp*I or *Taq*I site in Figure 6. This *Msp*I/*Taq*I linker is phosphorylated, contains a 3' blocking group on the 3' end of the top strand, and contains a bubble to prevent amplification of unwanted *Msp*I-*Msp*I, *Taq*I-*Msp*I, or *Taq*I-*Taq*I fragments. While the linker can ligate to itself in the phosphorylated state, these linker dimers will not amplify. Phosphorylation of the linker and use of a blocking group eliminates the potential artifactual amplification of unwanted *Msp*I-*Msp*I, *Taq*I-*Msp*I, or *Taq*I-*Taq*I fragments. T4 ligase attaches the *Drd*I and *Msp*I/*Taq*I linkers to their respective sites on the BAC DNA fragments with biochemical selection assuring that most sites contain linkers. The ligation product is PCR amplified using primers complementary to the linkers. After amplification is completed, dideoxy sequencing can be performed as described above.

Figure 7 describes the three levels of specificity in using the *DrdI* island approach.

Specificity of the *DrdI* Linker Ligations and Subsequent Sequencing Reactions.

The specificity of T4 thermostable DNA ligases is compared below in Table 3.

Table 3. Fidelity of T4 and different thermostable DNA ligases.



Ligase	Concentration (nM)	Initial Rate of C-G match (fmol/)	Initial Rate of T-G mismatch at 3'-end (fmol/)	Initial Rate of T-G mismatch at penultimate 3'-end (fmol/)	Ligation fidelity 1[a]	Ligation fidelity 2[b]
T4	0.5	1.4×10^{2}	2.8	7.1	5.0×10^{1}	1.9×10^{1}
T. th-wt	1.25	5.5×10^{1}	6.5×10^{-2}	2.9×10^{-1}	8.4×10^{2}	1.9×10^{2}
T. th-K294R	12.5	1.5×10^{2}	2.9×10^{-2}	3.8×10^{-1}	5.3×10^{3}	4.0×10^{2}
T. sp AK16D	12.5	1.3×10^{2}	2.5×10^{-2}	1.2×10^{-1}	5.2×10^{3}	1.1×10^{3}
Aquifex sp.	12.5	9.9×10^{1}	2.9×10^{-2}	2.6×10^{-1}	3.5×10^{3}	3.8×10^{2}

The reaction mixture consisted of 20 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 100 mM KCl, 10 mM DTT, 1 mM NAD^+, 20 µg/ml BSA, and 12.5 nM nicked DNA duplex substrates. T4 DNA ligase fidelity was assayed at 37 °C, Thermostable ligase fidelity was assayed at 65 °C. Fluorescently labeled products were separated on an ABI 373 DNA sequencer and quantified using the ABI GeneScan 672 software.

a: Ligation fidelity 1= Initial Rate of C-G match / Initial Rate of T-G mismatch at 3'-end.

b: Ligation fidelity 2= Initial Rate of C-G match / Initial Rate of T-G mismatch at penultimate 3'-end.

Both the thermostable and the T4 ligase show the highest degree of mismatch ligation for G:T or T:G mismatches. Consequently, by studying the fidelity of these reactions, the limits of mismatch discrimination may be determined.

While the thermostable ligases exhibit 10 to 100-fold greater fidelity than T4 ligase, the later enzyme is far more efficient in ligating 2 base overhangs. Therefore, ligation, in accordance with the present invention, should be performed using T4 ligase. There are three degrees of specificity: (i) ligation of the top strand requires perfect complementarity at the 3' side of the junction; (ii) ligation of the

bottom strand requires perfect complementarity at the 3' side of the junction; and (iii) extension of polymerase off the sequencing primer is most efficient if the 3' base is perfectly matched. All three of these reactions demonstrate 50-fold or greater discrimination if the match or mismatch is at the 3' end and 20-fold or greater discrimination if the match or mismatch is at the penultimate position to the 3' end.

How to interpret the results:

A computer simulation was performed on 4 known sequenced BAC clones from chromosome 7q31. The distribution of *Drd*I sites in these clones and their overhangs is shown in Figures 8-11. There are 38 non-palindromic *Drd*I sites in about 550 kb of DNA, or an average of 1 non-palindromic *Drd*I site per 15 kb.

The average 30-40 kb clone should be cut about three times with *Drd*I to generate non-palindromic ends. Again, palindromic ends are discounted, so the average clone needs to be a little bigger to accommodate the extra silent cuts and still get an average of 3 non-palindromic cuts. It should be noted, however, that as long as there are 2 or more *Drd*I sites which are singlets (i.e. present once in the clone) or doublets (present twice in the clone) in all of the clones to be aligned, such alignment can be successfully achieved. In the best case scenario, each of the overhangs is unique (i.e. a singlet), so 6 unique sequencing runs are generated, and these are connected in matched pairs (i.e. the sequence generated from the primers ending in AA is connected to the sequence generated from primers ending in TT), so about 3 x 1kb "*Drd*I islands" of sequence are somewhere within the 30-40kb flanked by the two 500-800 base-pair anchors.

Now if two random 30-40kb clones overlap, the chances are excellent that they will either run into each other on the ends, or, alternatively, 1 to 3 of the internal sequences will be identical. There will be a few case where two clones overlap and different internal 1kb sequences are obtained, because there is a small probability of having a *Drd*I polymorphism. However, these will simply add to the density of sequence which may run into or overlap with existing markers.

As shown in Figure 8, use of the *Drd*I approach in mapping the Met Oncogene in a BAC clone from the 7q31 chromosome identifies 12 *Drd*I sites within

the 171,905 bp shown. The overhangs and complements shown in the positions set forth in Figure 8 are based on the known sequence in GenBank. More particularly, there are TC and CA singlets and GG, GT, CT, and TT doublets (either in the overhang or its complement) for the *Drd*I islands. Since the sum of singlets and doublets is greater than or equal to 2, this fingerprint for the Met Oncogene in a BAC clone can be used to determine the positional relationship of this clone with respect to other clones in the library as described *infra*.

Figure 10 shows how the *Drd*I approach is used in mapping the HMG gene in a BAC clone from the 7q31 chromosome. Within the 165,608 bp shown, there are 11 *Drd*I sites with the known sequences used to identify the overhangs and complements in the positions set forth in Figure 10. More particularly, there are TT, GT, and GA singlets and CT and GG doublets (either in the overhang or its complement) for the *Drd*I islands. Since the sum of singlets and doublets is greater than or equal to 2, this fingerprint for the Met Oncogene in a BAC clone can be used to determine the positional relationship of this clone with respect to other clones in the library, as describe *infra*.

Figures 12 shows the use of the *Drd*I approach in mapping the Pendrin gene in a BAC clone from the 7q31 chromosome to identify 10 *Drd*I sites within the 97,943 bp shown. The overhangs and complements shown in the positions set forth in Figure 12 are based on the known sequence in GenBank. Specifically, there are 3 singlets (i.e. CC, TT, and GA), 1 doublet (i.e. AA), and 1 multiplet (i.e. CT) (either in the overhang or its complement) for the *Drd*I islands. Since the sum of singlets and doublets is greater than or equal to 2, this fingerprint for the Pendrin gene in a BAC clone can be used to determine the positional relationship of this clone with respect to other clones in the library, as described *infra*.

Figure 14 shows how the *Drd*I approach is used in mapping the alpha2(I) gene in a BAC clone from the 7q31 chromosome. There are 11 *Drd*I sites within the 116,466 bp with the known sequences used to identify the overhangs and complements shown in the positions set forth in Figure 14. There are 2 singlets (i.e. AG and GG) and 4 doublets (i.e. AA, TG, GT, and TC) (either in the overhang or its complement) for the *Drd*I islands. Since the sum of singlets and doublets is 2 or

greater, this fingerprint for the alpha2(I) gene can be used to determine the positional relationship of this clone with respect to other clones in the library, as described *infra*.

Two special cases need to be considered:

In the first case, the clone has no internal *DrdI* sites with non-palindromic ends. This will occur on occasion. Again, computer analysis on the four fully sequenced BAC clones (about 550 kb of DNA) showed two areas which would leave gaps in the cosmid contigs. This does not preclude overlapping such clones to larger superstructures (i.e. BACs and YACs).

The solution to this problem is to use a second enzyme with a comparable frequency in the human genome. By slightly modifying the procedure, 16 linker/primer sets may be used on split palindrome enzymes which generate a 3 base 3' overhang. Since the overhang is an odd number of bases, it is not necessary to exclude the palindromic two base sequences AT, TA, GC, and CG. To reduce the number of ligations from 64 (all the different possible 3 base overhangs) to 16, the linkers and primers are degenerate at the third position, i.e. end with NTC or NGC. As noted above, since there are 3 levels of specificity in the ligation and sequencing step, the third base degeneracy will not interfere with the fidelity of the reaction. With 3 base overhangs, multiplet sequences which are difficult to interpret may be teased apart by either: (i) using linkers and primers which lack the 3rd base degeneracy, or (ii) using sequencing primers which extend an extra base on the 3' end of the primer.

Of the 4 commercially available split palindrome enzymes which generate a 3 base 3' overhang, *BglI* (GCCNNNN^NGGC) and *Dra*III (CACNNN^GTG) are present at low enough frequencies to be compatible with *DrdI*. There are 60 *BglI* sites in about 550 kb of the four sequenced BAC clones, or an average of 1 *BglI* site per 9 kb. The frequency of the other split palindrome enzymes in human DNA are: *Dra*III (1 per 8 kb), *Alwn*I (1 per 4 kb), and *Pfl*MI (1 per 3 kb).

Although there are some type IIs enzymes which will allow the same 2 base overhang 3' ligation, they are not split palindromes and hence simultaneous cutting and ligation will only provide the sequence from one side. This can be an advantage for some enzymes, as described for *SapI* below.

Figures 8, 10, 12, and 14 show how the enzyme *BglI* can generate a 3 base 3' overhang which can be used in accordance with the present invention.

Figure 8 shows the use of the *BglI* approach in mapping the Met Oncogene in a BAC clone from the 7q31 chromosome. There are 16 *BglI* sites within the 171,905 bp shown with known sequences used to identify the overhangs and complements. More particularly, there are 5 singlets (i.e. the CT, TT, TG, TC, and CG overhangs) and 5 doublets (i.e. the TA, GG, CC, GA, and AG overhangs) (either in the overhang or its complement) for the *BglI* islands. Since the sum of the singlets and doublets is greater than or equal to 2, this fingerprint for the Met Oncogene in a BAC clone can be used to determine the positional relationship of this clone with respect to other clones in the library, as described *infra*.

Figure 10 shows the use of the *BglI* approach in mapping the HMG gene in a BAC clone from the 7q31 chromosome. Within the 165,608 bp shown, there are 12 *BglI* sites with known sequences used to identify the overhangs and complements in the positions set forth in Figure 9. Specifically, there are 5 singlets (i.e. the GT, AA, AC, GC, and CC overhangs) and 4 doublets (i.e. the AG, TC, TT, and CA overhangs) (either in the overhang or its complement) for the *BglI* islands. Since the sum of the singlets and doublets is greater than or equal to 2, this fingerprint for the Met Oncogene in a BAC clone can be used to determine the positional relationship of this clone with respect to other clones in the library, as described *infra*.

Figure 12 shows the use of the *BglI* approach in mapping the Pendrin gene in a BAC clone from the 7q31 chromosome to identify the 17 *BglI* sites within the 97,943 bp shown. The overhangs and complements shown in the positions set forth in Figure 10 are based on known sequences. Specifically, there is 1 singlet (i.e. the TC overhang) and 5 doublets (i.e. TA, GT, CC, TT, and AA overhangs) (either in the overhang or its complement) for the *BglI* islands. Since the sum of the singlets and doublets is greater than or equal to 2, this fingerprint for the Pendrin gene in a BAC clone can be used to determine the positional relationship of this clone with respect to other clones in the library, as described *infra*.

Figure 14 shows how the use of the *BglI* approach is used in mapping the alpha2(I) gene in a BAC clone from the 7q31 chromosome. There are 15 *BglI* sites within the 116,466 bp with known sequences used to identify the overhangs and

complements shown in the positions set forth in Figure 11. There are 4 singlets (i.e. the AA, TT, GC, and GG overhangs) and 7 doublets (i.e. the TA, GA, CG, TC, AA, CC, and AC overhangs) (either in the overhang or its complement) for the *BglI* islands. Since the sum of the singlets and doublets is greater than or equal to 2, this fingerprint for the alpha2(I) gene can be used to determine the positional relationship of this clone with respect to other clones in the library, as described *infra.*

Similarily, Figures 9, 11, 13, and 15 show how the enzyme *SapI* can also generate 3 base 3' overhangs in accordance with the present invention. Figure 16 is a schematic drawing showing the sequencing of *BglI* islands in random BAC clones in accordance with the present invention. This is largely the same as the embodiment of Figure 7, except that a different enzyme is used. In this embodiment, individual BAC clones are cut with the restriction enzymes *BglI* and *MspI* in the presence of linkers and T4 ligase. As in Figure 7, the linker for the *BglI* site is phosphorylated and contains a 3' three base overhang (e.g., a 3' NAC overhang). A separate linker is used for the *MspI* site which replaces the portion of the BAC clone DNA to the right of the *MspI* site in Figure 7. The *MspI* linker is not phosphorylated and contains a bubble (i.e. a region where the nucleotides of this double stranded DNA molecule are not complementary) to prevent amplification of unwanted *MspI*-*MspI* fragments. The T4 ligase binds the *BglI* and *MspI* linkers to their respective sites on the BAC clone DNA with biochemical selection assuring that most sites contain linkers.

After the different linkers are ligated to the fragments of DNA produced by *BglI* digestion to form a phosphorylated site containing, in the case of Figure 16, a 3' NAC overhang, the T4 ligase and the restriction enzymes (i.e. *BglI* and *MspI*) are inactivated at 65°C to 98°C, preferably 95°C, for 2 minutes to 20 minutes, preferably 5 minutes. As shown in Figure 16, the ligation product is amplified using a PCR procedure under the conditions described above. For the linker depicted, one amplification primer has a 3' AC overhang and nucleotides 5' to the overhang which makes the primer suitable for hybridization to bottom strand of the ligation product for polymerization in the 3' to 5' direction. Amplification primers adapted to hybridize to the ligation products formed from the other linkers are similarly

provided. As described with reference to Figure 6, PCR amplification is carried out using primers with ribose U instead of dT, adding dNTPs and *Taq* polymerase, adding NaOH, and heating at 85°C to 98°C, preferably 95°C, for 2 minutes to 20 minutes, preferably 5 minutes to inactivate any unused primer.

After amplification is completed and the amplification product is neutralized and diluted, dideoxy sequencing can be conducted in substantially the same manner as discussed above with reference to Figure 1. If necessary, a separate dideoxy sequencing procedure can be conducted using a sequencing primer which anneals to the *Msp*I site linker. This is useful to generate additional sequence information associated with the *BglI* island.

Another departure from the schematic of Figure 5 is that, in the scheme of Figure 16, a separate linker ligation procedure is carried out with the portion of the BAC clone on the left side of Figure 16. The primer utilized in this procedure is phosphorylated and ends with a 3' NTA overlap sequence.

Figure 17 is a schematic drawing showing the sequencing of *Sap*I islands in random BAC clones in accordance with the present invention. This is largely the same as the embodiment of Figure 5, except that a different enzyme is used. In this embodiment, individual BAC clones are cut with the restriction enzymes *Sap*I and *Msp*I in the presence of linkers and T4 ligase. As in Figure 5, the linker for the *Sap*I site is phosphorylated and contains a 3' three base overhang (e.g., a 3' NUG overhang). A separate linker is used for the *Msp*I site which replaces the portion of the BAC DNA to the right of the *Msp*I site as in Figure 5. The *Msp*I linker is not phosphorylated and contains a bubble (i.e. a region where the nucleotides of this double stranded DNA molecule are not complementary) to prevent amplification of unwanted *Msp*I-*Msp*I fragments. The T4 ligase binds the *Sap*I and *Msp*I linkers to their respective sites on the BAC DNA with biochemical selection assuring that most sites contain linkers.

After the different linkers are ligated to the fragments of DNA produced by *Sap*I digestion to form a phosphorylated site containing, in the case of Figure 5, a 3' NUG overhang, the T4 ligase and the restriction enzymes (i.e. *Sap*I and *Msp*I) are inactivated at 65°C to 98°C, preferably 95°C, for 2 minutes to 20 minutes, preferably 5 minutes. As shown in Figure 15, the ligation product is amplified using a

PCR procedure under the conditions described above. For the linker depicted, one amplification primer has a 3' NTG overhang and nucleotides 5' to the overhang which makes the primer suitable for hybridization to the bottom strand of the ligation product for polymerization in the 3' to 5' direction. The other sequencing primer, for the linker depicted in Figure 17, has a 5' CA overhang which makes this primer suitable for hybridization to the top strand of the ligation product for polymerization in the 5' to 3' direction. Amplification primers adapted to hybridize to the ligation products formed from the other linkers are similarly provided. As described with reference to Figure 4, PCR amplification is carried out using primers with ribose U instead of dT, adding dNTPs and *Taq* polymerase, adding NaOH, and heating at 85°C to 98°C, preferably 95°C, for 2 minutes to 20 minutes, preferably 5 minutes to inactivate any unused primer.

After amplification is completed and the amplification product is neutralized and diluted, dideoxy sequencing can be conducted in substantially the same manner as discussed above with reference to Figure 1. If necessary, a separate dideoxy sequencing procedure can be conducted using a sequencing primer which anneals to the *Msp*I site linker. This is useful to generate additional sequence information associated with the *Sap*I island.

In a second case, the clone has two *Drd*I sites with the same 3' overhangs. Thus, the sequencing reads have two bases at each position. The probability of NOT having an overlap is 6/6 x 5/6 x 4/6 = 20/36 = 0.55. So the probability of *having* an overlap is 1- 0.55 = 0.45, or about every other clone. At first glance, this is may appear to cause a problem, but, in fact, it is very useful. Rather than discarding these reads, on average every 4th base will be the same in both reads and, thus, clearly distinguishable. Thus, a read of this form will be entered into the database as such: G---A-----C--C----T---AA-----T, etc. The current computer programs which look for overlap examine 32 bases at a time, which is essentially unique in the genome, so the first 128 bases of a double-primed sequencing run creates a unique "signature". This can be checked against the existing sequences in the database as well as against the *Drd*I sequences generated from other clones. It will line up either with a single read (i.e. when only one of the sites overlaps) or as an identical double read (i.e. when both sites overlap). It is reasonably straightforward to

do a "subtraction" of one sequence from the double sequence to obtain the "hidden" sequence.

Evaluation of the BAC clones reveals a few instances where the same overhang would appear in two *Drd*I sites from neighboring random 30-40kb clones. This requires that additional neighboring clones are found in a larger contig. If a region remains intractable to analysis, because there are too many *Drd*I sites with the identical overhangs, alternative enzymes *Bgl*I and *Dra*III may be used. A second solution to sequencing reads which are difficult to interpret is to use four separate sequencing reactions with primers containing an additional base on the 3' end, as depicted at the bottom of Figure 1.

One advantage about generating *Drd*I islands is the format of the data. The sequence information always starts at the same position. Thus, the computer programs can be vastly simpler than previous lineup algorithms. A computer program sets up bins to score identity. For example:

```
SEQ. ID. No. 1.
GATTCGATCGTAGCGTGTAGCAAGTAGCTAATTCGATCCA
                        |
GATTCGATCGTAGCGTGTAACAAGTAGCTAATTCGATCCA
SEQ. ID. No. 2.
```

i.e. 39/40 match, score as an overlap (with an SNP at position 20).

Further simplifying the computer analysis, sequence information in the *Drd*I analysis is generated in 12 separate sets, corresponding to each overhang, and these sets are virtually exclusive. The probability of having a polymorphism right at the 2 base 3' overhang is very small (about 2 in 1,000), and, even if the polymorphism does occur, it will make two sequences jump to new bins, making it very easy to double-check existence of such polymorphisms.

The above scheme has a built in redundancy, because each forward sequence on a *Drd*I site is matched to a reverse sequence. It may be more cost effective to ligate primers which give only one sequence read off a *Drd*I site. The above example just doubles the probability of obtaining a sequence which overlaps with either known STS's or with the two 500 base-pair sequences from the end of the clone.

III. Singlet and Doublet *DrdI* Island Approach

Extending the *DrdI* island approach to allow for alignment of BACs.

On average, a given BAC will contain 2-3 unique sequences (called "singlets"), 2-4 sequences which are the consequence of two overlapping runs (called "doublets") and 0-1 sequences which are the consequence of three or more overlapping runs, which may be un-interpretable multisequences. In order to construct BAC clone overlaps, it is necessary to have at least two readable (doublets or singlets) sequencing runs for a given BAC.

The probabilities of obtaining two readable sequencing runs from a BAC clone containing from 2 to 20 *DrdI* sites are as follows.

A given restriction site may appear multiple times in a given BAC clone. Therefore, it is necessary to determine the frequency of unique and doubly represented restriction sites in a BAC clone. Sites which appear only once in a BAC clone will generate a clean sequence and will be called singlets in the calculations. Sites which appear exactly twice should still reveal useful sequencing data once every four bases on average and will be known as doublets in the calculations.

The *DrdI* enzyme generates a degenerate 2 base 3' overhang. After eliminating palindromic sequences for the degenerate positions, there are 6 different overhangs which can be ligated after digestion of a BAC with *DrdI*.

The *SapI* and *BglI* enzymes generate degenerate 3 base 5' and 3' overhangs, respectively. 16 possible tails can be picked to ensure specific ligation and to simplify the complexity of the sequencing reactions.

Below is an analysis of the possible ways that these restriction enzyme sites can be distributed in BAC clones containing between 1 and 36 restriction sites. From the representative BAC clones, the (non-palindromic overhang) *DrdI* site appears from 8-10 times, the *BglI* site appears from 12-17 times, and the *SapI* site appears from 12 to 25 times in human DNA. Note that the *BglI* site is used on both sides of the cut, so for the calculations below, one doubles the number of BglI sites in the BAC when calculating "N".

The probability of each site is p = 1/n where n = 6 for *Drd*I and n = 16 for *Sap*I or *Bgl*I.

For a given restriction sequence **R**, the probability of a given site not being **R** is q.

$q = 1 - p$

$= 1 - 1/n.$

The probability of all **N** sites in a given BAC not being the sequence **R** is P(absent) = q^N.

The probability of **R** appearing once and only once in **N** sites in a given BAC is:

$P(\text{singlet}) = p \times q^{(N-1)} \times N$

The probability of **R** appearing twice and only twice in **N** sites in a given BAC is:

$P(\text{doublet}) = p^2 \times q^{(N-2)} \times \text{Comb}(N,2)$

$= p^2 \times q^{(N-2)} \times (N)(N-1)/2$

Where Comb(N,n) is the number of ways that **n** items can be picked from a set of **N** available items.

The probability that at least one of the 6 possible *Drd*I sites is a singlet:

$P(\text{at least one singlet}) = 1 - (1-P(\text{singlet}))^6$

The probability that at least one of the 16 possible *Sap*I or *Bgl*I sites is a singlet:

$P(\text{at least one singlet}) = 1 - (1-P(\text{singlet}))^{16}$

The probability that at least one of the 6 possible *Drd*I sites is either a singlet or a doublet is:

Psd = P(singlet) + P(doublet)

$P(\text{at least one singlet or doublet}) = 1 - (1-Psd)^6$

The probability that at least one of the 16 possible *Sap*I or *Bgl*I sites is either a singlet or a doublet is:

Psd = P(singlet) + P(doublet)

$P(\text{at least one singlet or doublet}) = 1 - (1-Psd)^{16}$

The probability of one and only one singlet or doublet for *Drd*I is:

$P(\text{exactly one singlet or doublet}) = 6 \times Psd \times (1 - Psd)^5$

$P(\text{exactly one singlet}) = 6 \times P(\text{singlet}) \times (1 - P(\text{singlet}))^5$

The probability of one and only one singlet or doublet for *Sap*I or *Bgl*I is:

$P(\text{exactly one singlet or doublet}) = 16 \times Psd \times (1 - Psd)^{15}$

$P(\text{exactly one singlet}) = 16 \times P(\text{singlet}) \times (1 - P(\text{singlet}))^{15}$

For the BAC clones to be informative for constructing overlapping contigs, one needs at least two readable sequences per clone. Calculations are provided for at least two singlets or doublets, or the more stringent requirement of at least two singlets.

The probability of at least two singlets or doublets for *Drd*I is:
P(at least two singlets or doublets) = P(at least one singlet or doublet) - P(exactly one singlet or doublet)

$$= 1 - (1\text{-Psd})^6 - 6 \times \text{Psd} \times (1 - \text{Psd})^5$$

The probability of at least two singlets for *Drd*I is:
P(at least two singlets) = P(at least one singlet) - P(exactly one singlet)

$$= 1 - (1\text{-P(singlet)})^6 - 6 \times \text{P(singlet)} \times (1 - \text{P(singlet)})^5$$

The probability of at least two singlets or doublets for *Sap*I or *Bgl*I is:
P(at least two singlets or doublets) = P(at least one singlet or doublet) - P(exactly one singlet or doublet)

$$= 1 - (1\text{-Psd})^{16} - 16 \times \text{Psd} \times (1 - \text{Psd})^{15}$$

The probability of at least two singlets for *Sap*I or *Bgl*I is:
P(at least two singlets) = P(at least one singlet) - P(exactly one singlet)

$$= 1 - (1\text{-P(singlet)})^{16} - 16 \times \text{P(singlet)} \times (1 - \text{P(singlet)})^{15}$$

(Note: For small values, the charts below are not completely accurate.)

Using these equations, for *Drd*I the probabilities are:

N	P(absent)	P(singlet)	P(doublet)	P(sd)	P(at least two singlets or doublets)	P(at least two singlets)
1	0.83333	0.16667	0.00000	0.16667	0.26322	0.26322
2	0.69444	0.27778	0.02778	0.30556	0.59175	0.53059
3	0.57870	0.34722	0.06944	0.41667	0.79174	0.67569
4	0.48225	0.38580	0.11574	0.50154	0.89207	0.74399
5	0.40188	0.40188	0.16075	0.56263	0.93897	0.76963
6	0.33490	0.40188	0.20094	0.60282	0.96032	0.76963
7	0.27908	0.39071	0.23443	0.62514	0.96946	0.75200
8	0.23257	0.37211	0.26048	0.63259	0.97213	0.72083
9	0.19381	0.34885	0.27908	0.62793	0.97048	0.67876
10	0.16151	0.32301	0.29071	0.61372	0.96501	0.62813
11	0.13459	0.29609	0.29609	0.59219	0.95532	0.57134
12	0.11216	0.26918	0.29609	0.56527	0.94059	0.51093
13	0.09346	0.24301	0.29161	0.53461	0.91981	0.44939
14	0.07789	0.21808	0.28351	0.50159	0.89211	0.38901
15	0.06491	0.19472	0.27260	0.46732	0.85690	0.33166
16	0.05409	0.17308	0.25962	0.43270	0.81412	0.27875
17	0.04507	0.15325	0.24520	0.39845	0.76430	0.23117
18	0.03756	0.13522	0.22987	0.36509	0.70850	0.18936
19	0.03130	0.11894	0.21410	0.33304	0.64826	0.15335
20	0.02608	0.10434	0.19824	0.30258	0.58537	0.12290
21	0.02174	0.09129	0.18259	0.27388	0.52173	0.09756
22	0.01811	0.07970	0.16737	0.24707	0.45911	0.07677
23	0.01509	0.06944	0.15276	0.22220	0.39906	0.05994
24	0.01258	0.06038	0.13887	0.19925	0.34280	0.04646
25	0.01048	0.05241	0.12579	0.17820	0.29121	0.03578
26	0.00874	0.04542	0.11356	0.15899	0.24480	0.02739
27	0.00728	0.03931	0.10221	0.14152	0.20376	0.02085
28	0.00607	0.03397	0.09172	0.12569	0.16805	0.01580
29	0.00506	0.02932	0.08210	0.11142	0.13742	0.01192
30	0.00421	0.02528	0.07330	0.09858	0.11148	0.00896
31	0.00351	0.02177	0.06530	0.08706	0.08977	0.00670
32	0.00293	0.01872	0.05804	0.07677	0.07180	0.00500
33	0.00244	0.01609	0.05149	0.06758	0.05706	0.00372
34	0.00203	0.01381	0.04559	0.05940	0.04509	0.00276
35	0.00169	0.01185	0.04029	0.05214	0.03544	0.00204
36	0.00141	0.01016	0.03555	0.04571	0.02771	0.00151

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Using these equations, for *Sap*I or *Bgl*I the probabilities are:

N	P(absent)	P(singlet)	P(doublet)	P(sd)	P(at least two singlets or doublets)	P(at least two singlets)
1	0.93750	0.06250	0.00000	0.06250	0.26411	0.26411
2	0.87891	0.11719	0.00391	0.12109	0.59371	0.57480
3	0.82397	0.16479	0.01099	0.17578	0.79985	0.76694
4	0.77248	0.20599	0.02060	0.22659	0.90679	0.87145
5	0.72420	0.24140	0.03219	0.27359	0.95777	0.92673
6	0.67893	0.27157	0.04526	0.31684	0.98104	0.95624
7	0.63650	0.29703	0.05941	0.35644	0.99146	0.97240
8	0.59672	0.31825	0.07426	0.39251	0.99610	0.98156
9	0.55942	0.33565	0.08951	0.42516	0.99818	0.98692
10	0.52446	0.34964	0.10489	0.45453	0.99912	0.99016
11	0.49168	0.36057	0.12019	0.48076	0.99956	0.99217
12	0.46095	0.36876	0.13521	0.50397	0.99977	0.99342
13	0.43214	0.37452	0.14981	0.52433	0.99987	0.99419
14	0.40513	0.37812	0.16385	0.54198	0.99993	0.99463
15	0.37981	0.37981	0.17725	0.55706	0.99995	0.99483
16	0.35607	0.37981	0.18991	0.56972	0.99997	0.99483
17	0.33382	0.37833	0.20178	0.58010	0.99998	0.99466
18	0.31296	0.37555	0.21281	0.58836	0.99998	0.99432
19	0.29340	0.37163	0.22298	0.59462	0.99999	0.99382
20	0.27506	0.36675	0.23227	0.59902	0.99999	0.99313
21	0.25787	0.36101	0.24068	0.60169	0.99999	0.99225
22	0.24175	0.35457	0.24820	0.60277	0.99999	0.99112
23	0.22664	0.34752	0.25485	0.60236	0.99999	0.98972
24	0.21248	0.33996	0.26064	0.60060	0.99999	0.98801
25	0.19920	0.33199	0.26560	0.59759	0.99999	0.98593
26	0.18675	0.32369	0.26975	0.59344	0.99999	0.98342
27	0.17508	0.31514	0.27312	0.58825	0.99998	0.98041
28	0.16413	0.30638	0.27574	0.58212	0.99998	0.97684
29	0.15387	0.29749	0.27766	0.57515	0.99997	0.97264
30	0.14426	0.28851	0.27890	0.56741	0.99997	0.96771
31	0.13524	0.27950	0.27950	0.55900	0.99996	0.96199
32	0.12679	0.27048	0.27950	0.54998	0.99994	0.95539
33	0.11886	0.26150	0.27894	0.54044	0.99992	0.94783
34	0.11144	0.25259	0.27785	0.53043	0.99989	0.93924
35	0.10447	0.24377	0.27627	0.52003	0.99985	0.92955
36	0.09794	0.23506	0.27424	0.50929	0.99980	0.91869

Graphs showing the probabilities of two or more singlets or doublets of *Drd*I, *Sap*I, or *Bgl*I sites in BACs containing from 2 to 36 sites are shown in Figure 17A.

For the average of 8-12 non-palindromic *Drd*I sites per BAC clone, the probability is from 94%-97% of containing at least two readable (singlet or doublet)

sequences. For the same clones, from 51%-72% will contain at least two singlet sequences, making alignment even easier for those clones.

Thus, the overwhelming majority of BAC clones will contain at least two readable (doublets or singlets) sequencing runs. Contigs may be constructed off *DrdI* doublet sequencing runs since two doublet runs may be used to determine BAC overlap, even if individual singlet sequences are unknown. Further, since the BAC library will represent a 5-fold coverage of the genome, sequences which were buried within three overlapping runs in one BAC clone will be represented as either singlets of doublets in neighboring BAC clones. Surprisingly, the doublet data will even allow for mapping virtually all *DrdI* islands onto the BAC clones.

How to collect the data:

In the past "Gemini proteins" (i.e. proteins with duplicated domains) were constructed. When using a sequencing primer which hybridizes to the duplicated region, one obtains a sequencing run with a single read which turns into a double read as the sequencing reaction extends past the duplicated region. Bands were clearly visible for both sequences and the precise sequence could be determined by subtracting the "known" sequence from the doublet sequence. New automated DNA sequencing machines give excellent peak to peak resolution and would be able to read doublet and even triplet sequences for hundreds of bases.

How to interpret the results:

A computer simulation was performed on 4 known sequenced BAC clones from chromosome 7, and each clone generated at least 5 readable sequences. A computer simulation of *DrdI* site sequences was performed on the first 5 such sites in BAC RG253B13. The first 80 bp of sequence from each of these positions was compared for either "concordant" or "discordant" alignment tests for a doublet sequence.

To understand the power of aligning *DrdI* sites, it is important to realize there are only about 200,000 to 300,000 *DrdI* sites in the human genome. Further, since these are being sequenced in 6 different sets, there are about 35,000 to

50,000 *Drd*I sites in a given set. Thus, to distinguish a given sequence from others, it must be unique at only one in 50,000 (not one in 3 billion) sites.

A key advantage for generating *Drd*I islands is the format of the data. The sequence information always starts at the same position. The GTC half of the *Drd*I site is retained in the sequencing read, thus assuring that the sequences are always aligned correctly (see e.g. Figure 18 where sequences 1, 2, 3, 4, and 5 (i.e. SEQ. ID. Nos. 3, 4, 5, 6, and 7, respectively) are aligned at the GTC motif). All the sequences have the same orientation. There is no need to compare multiple alignments or try the reverse sequence for alignment. Thus, computer programs can be vastly simpler than previous lineup algorithms.

When comparing two singlet sequences, the uniqueness is determined for any stretch of 8 bases (i.e. $4^8 = 65,536$). When comparing a doublet sequence with a singlet sequence, the uniqueness may be determined either (1) by scoring identity at 8 bases in the doublet sequence with the singlet sequence (represented by vertical bars (i.e. |) in Figure 18), or (2) by scoring 16 bases (i.e. 2^{16}=65,536) where the singlet sequence is consistent with either of the bases in the doublet at that position (represented by a comma in Figure 18 (i.e. ,).

For example, in Figure 18, when analyzing the doublet to singlet concordant sequences, the vertical line (i.e. |) indicates identity where the corresponding base for the doublet and for the singlet are all the same. On the other hand, the comma (i.e. ,) indicates consistency in that one of the bases in the doublet is the same as the corresponding base in the singlet. In this example, there is concordance (i.e . the sequences must match), because the number of bases, aside from the GTC motif, which are identical (i.e. 12) is greater than 8 and which are consistent (i.e. 63) exceeds 16. On the other hand, with regard to the doublet to singlet discordant sequences, there are no vertical lines (i.e. |) or commas (i.e. ,) and, as indicated by the Xs, there are numerous bases where neither base from the doublet can match the corresponding base in the singlet. As a result, the doublet and the singlet cannot be from the same clone (i.e. they are discordant).

When comparing a doublet read to another doublet read, the sequences will contain a shared concordant read if there are at least 16 bases where either doublet sequence has an identical base which is consistent with one or the other of the

two bases represented in the other doublet sequence. For example, in the concordance comparison of a doublet in a first clone to a doublet in a second clone of Figure 18, the vertical line (i.e. |) indicates identity where both bases of one doublet are the same as one corresponding base in the other doublet. On the other hand, the comma (i.e. ,) indicates consistency in that there are 2 different corresponding bases in one doublet which are the same as the corresponding bases in the other doublet. For example, in Figure 18, there is concordance, because, aside from the GTC motif, the number of bases with identity (i.e. 26) (as indicated by |) added to the number of bases with consistency (i.e. 17) (as indicated by a comma) (i.e. 26 + 17 = 43) exceeds 16. Turning to doublet to doublet analysis for discordance in Figure 18, there are no vertical lines or commas, but, at several bases, there are Xs, indicating that neither base from one doublet matches a corresponding base from the other doublet. This is, perhaps, the most striking example of the power of this approach in that it easily shows if two multiple bases do not overlap. In a random comparison of a doublet and a singlet sequence, there are only 3 positions which are identical (|), and 38 which are discordant (X). When comparing different doublets with one another, there are 12 discordant sites where one doublet has a single base (X), and 5 discordant sites where all four bases were present (two from one doublet, two from the other doublet; x). For simplicity, positions where more than two bases are read will not be considered, even though those positions are still informative.

Figure 18 also shows doublet to triplet analyses for concordant and discordant sequences. These procedures are carried out in substantially the same fashion as the doublet to doublet analysis described above. However, the vertical line (i.e. |) now indicates identity where both bases of one doublet are the same as one corresponding base in the triplet or all bases of the triplet are the same as one corresponding base in the doublet. On the other hand, the comma (i.e. ,) indicates consistency in that there are 2 different corresponding bases in the doublet which are the same two of the corresponding bases in the triplet.

Again, the sequences will contain a shared concordant read if there are at least 16 cases where either doublet or triplet sequence has an identical base which is consistent with one or the other of the two bases represented in the other sequence. For example, in the alignment of cordant sequences for the doublet to triplet in

Figure 18, there are 12 such positions in the first 80 bp. However, there are also 14 positions where the two reads have the same two bases at that positions, bringing the total concordant positions to 26, well in excess of the 16 positions required. Comparing a doublet with a triplet yielded 11 discordant sites. The prediction is that one SNP will be observed every 1,000 bases, so single base discordance representing SNPs will be rare but also easily distinguished from the average of 10 to 40 discordant sites when comparing doublets with triplet, doublet, and singlet sequences.

Thus, in as few as 80 bases of sequence, one can easily discern if there is a common or discordant *Drd*I sequence within the two reads which are being compared, when the two reads contain a singlet, doublet, or even a triplet.

Using smaller representational fragments as an alternative approach to alignment of BACs

The previous section described an approach to interpret singlet, doublet, and triplet sequences generated from representations of individual BAC clones using as few as 80 bases of sequence information. The assumption was made that when more than one fragment is generated from a given representation (i.e. *Drd*I site AA overhang), then those fragments would be present in about equal amounts. Further, the above approach requires specialized software to interpret a sequencing read where more than one base is called at a given position. As an alternative to deconvoluting doublet and triplet sequencing runs, other enzymes may be used to create short representational fragments. Such fragments may be differentially enriched via ultrafiltration to provide dominant signal, or, alternatively, their differing length provides unique sequence signatures on a full length sequencing run, such that unique sequences for more than one fragment can be interpreted on a single sequencing lane.

For human DNA within BACs, *Mse*I can be substituted for *MspI/Taq*I, resulting in generation of much shorter representational fragments (Figure 19 and Figure 20). Bubble linkers for MspI/*Taq*I on one hand and for *Mse*I on the other hand are disclosed in Table 4.

Table 4. New *Msp*I/*Taq*I and *Mse*I bubble linkers.

New *Msp*I/*Taq*I linkers

MTCG225 5' GAC ACG TCA CGT CTC GAG TCC TA 3'
(SEQ. ID. No. 8)

MTCGO326R 3' Bk-TGC AGT GCA ACA CTC AGG ATGC 5'
(SEQ. ID. No. 9)

MTCG225 5' GAC ACG TCA CGT CTC GAG TCC TA 3'
(SEQ. ID. No. 10)

MTCGp326R 5' pCGT AGG ACT CAC AAC GTG ACG T - Bk
(SEQ. ID. No. 11)

MTCGO326R 5' CGT AGG ACT CAC AAC GTG ACG T - Bk
(SEQ. ID. No. 12)

MTCG227 5' GAC ACG TCA CGT CTC GAG TCC TsAsC 3'
(SEQ. ID. No. 13)

MTCG228 5' GAC ACG TCA CGT CTC GAG TCC TAC 3'
(SEQ. ID. No. 14)

New *Mse*I linkers (*Mse*I site = TTAA)

MSTA275 5' GAC ACG TCA CGT CTC GAG TCC TC 3'
(SEQ. ID. No. 15)

MSTAO276R 3' Bk-TGC AGT GCA ACA CTC AGG AGAT 5'
(SEQ. ID. No. 16)

MSTA275 5' GAC ACG TCA CGT CTC GAG TCC TC 3'
(SEQ. ID. No. 17)

MSTAp276R 5' pTAG AGG ACT CAC AAC GTG ACG T - Bk
(SEQ. ID. No. 18)

MSTAO276R 5' TAG AGG ACT CAC AAC GTG ACG T - Bk
(SEQ. ID. No. 19)

MSTA278 5' GAC ACG TCA CGT CTC GAG TCC TCT AA 3'
(SEQ. ID. No. 20)

*Mse*I cleaves human genomic DNA approximately every 125 bp. In contrast, when using *Msp*I/*Taq*I as the second enzyme, the average size fragment is greater than 1,000 bp. Many of the larger fragments (i.e. greater than 2,000 bp) will not amplify as well as smaller fragments in a representation, i.e. they will be lost to the sequencing gel. Therefore, in a *Drd*I-*Mse*I representation, the number of unique fragments lost during PCR amplification may be greatly reduced. This can increase the number of amplified fragments per BAC and can facilitate alignment of BACs.

*Drd*I representations of individual BACs can be used to link BACs together to form contigs. For BACs that generate a doublet sequence, "singlet" sequence information can still be obtained as long as the fragments are of different lengths. For example, an AG *Drd*I/*Mse*I representation of BAC RG253B13 results in two fragments of length 115 and 353 bases. Sequencing of these two fragments simultaneously will result in two distinct regions of sequence. The first region (approx. 1-141 bases) will consist of an overlap sequence in which sequence information from both fragments will be observed. The last 25 bases of this sequence will be the linker adapter sequence on the *Mse*I adapter. Thus, one can easily distinguish when the shorter fragment "ends" on the sequencing run. In all likelihood, it will also be more abundant and, hence, provide a stronger signal for those bases which were derived from that shorter fragment. If this stronger signal is not sufficient to recognize the unique sequence, then ultrafiltration (i.e. use of Amicon filters YM30 and YM125 (made by Millipore, Danvers, MA)) may be used to enrich for "smaller" vs. "larger" fragments. The second region (approx. 141-353 bases) will consist only of sequence information from the longer fragment. Therefore, for any doublet in which the fragments are of different length, a "singlet" sequence will be generated for the non-overlapping region of the longer fragment. This non-overlapping region of the doublet can be utilized as a "singlet" in order to overlap BACs. A minimum of 8 unique bases for a given distance from the *Drd*I site is sufficient to uniquely identify the sequence in the human genome, because the *Drd*I site provides an additional 6 + 2 = 8 bases of unique sequence, bringing the total to 16 bases.

How to align the BAC clones to create a complete contig of the entire human genome.

As mentioned earlier, there are only about 200,000 to 300,000 *Drd*I sites in the human genome. Since these are being sequenced in 6 different sets, there are about 35,000 to 50,000 *Drd*I sites in a given set. Alignment of the BAC clones is a simple process of constructing contigs *in each of the 6 sets*.

Consider creating contigs in the sequencing set whose linker primer ends in "GG". Suppose a given BAC =B1 clone contains a doublet sequence of #1 & #2. By searching the database one finds a second BAC =B2 clone containing a doublet sequence of #2 & #3. This implies that BAC clones B1 and B2 overlap, and further the order of the *Drd*I islands are #1, #2, and #3. (The approach for determining individual sequence runs #1, #2, and #3 are explained below.) Consider then additional BACs: B3 with islands #3, #4, and #5, B4 with #4 & #6, B5 with #6, and B7 with #6 & #7. Then the BAC clone overlap is B1-B7 and the sequences are in the order: #1, #2, #3, #5, #4, #6, #7. In other words, the *Drd*I islands not only line up the BAC clone overlaps, they also provide the order they appear in the linear sequence.

How frequent are the individual members of a set? With one non-palindromic *Drd*I site every 10-15 kb, the average distance between two *Drd*I sites with the same dinucleotide overhang sequence is 60 to 75 kb, or on average one to two such sites per BAC clone. Computer simulation on four BAC clones demonstrated 2 duplex sites separated by less than 25 kb, 5 duplex sites separated by between 25 kb and 50 kb, 2 duplex sites separated by between 50 kb and 75 kb, and 2 duplex sites greater than 75 kb apart. Thus, a 5-fold coverage of a region of DNA will create BAC clones with an average of two same overhang sites per BAC clone, but many such sites will be represented as either singlet or doublet reads in neighboring overlapping BAC clones.

On a rare occasion, a long stretch of human DNA will lack a *Drd*I site with a given dinucleotide overhang (i.e. GG), such that even larger BAC clones of 175-200 kb would not include two such sites. However, the BAC clone contigs are being pieced together using *six* sets of *Drd*I sequence information. This is akin to using six different restriction enzymes to create a restriction map of pBR322. Thus, a

"gap" in the contig is easily filled using sequence information from one of the other 5 sets. The average BAC of 8-12 *Drd*I sites contains sequence information ranging from 4 to all 6 of the different contig sets. Thus, by combining the contig building among the 6 different sets, the entire genome contig can be built.

Using the *Drd*I island database to obtain unique singlet sequences from overlapping doublet and triplet BAC clones.

When BAC overlaps are found, the data may be immediately used to deduce unique singlet sequences at essentially all of the *Drd*I sites. As the simplest case, when comparing a doublet with a singlet sequence, subtraction of the singlet sequence will reveal the other singlet in the doublet sequence. In most cases, a doublet will be represented again as a singlet in a neighboring BAC. In some cases, two or three doublets will be connected in a series. Even one singlet at the end of a string of doublets may be used to deduce the unique sequences of the individual *Drd*I islands.

Remarkably, just three overlapping doublets may be used to determine all four individual singlet sequences. For example, as shown in Figure 17, 4 unique singlet *Drd*I sequences from 2 overlapping doublet BAC clone sequences are obtained by aligning them as shown and comparing the corresponding bases. The common sequence between two doublets will either be identical, i.e. AA compared with AA (S), the same in one doublet allowing assignment, i.e. AA compared with AC indicates the common base is "A" (s), different among the doublets, also allowing assignment, i.e. AG compared with AC indicates the common base is "A" (d), or indeterminate, i.e. AC compared with AC does not reveal the base (i). On average, 3 out of every 4 positions will allow assignment of the common sequence base. Based upon this analysis, the sequence common in each doublet can be determined with a nucleotide at each location receiving an S, s, or d designation. In this manner, a sequence is identified with locations having the i designation being assigned alternative bases. Figure 21 shows how the sequences for #2 and #3 are determined in this fashion. This information can then be used to compare the consensus sequences of #2 and #3 from which one can determine the overlap. With only 2 indeterminant

bases, the sequences for #2 and #3 can be found. Sequence information for #1 and #4 can then be obtained.

The same analysis may be applied to alignment of one of the doublets with another neighboring doublet (or even triplet). See Figure 22. Although the sequence which is common between these sets is different from the original doublet comparison, the two consensus sequences may now be compared with the original doublet sequencing run. The probability that the indeterminate sequence in one sequence is at the same position as the other sequence is 1/4 x 1/4 = 1/16 for the doublet-doublet-doublet comparison and 1/4 x 7/16 = 7/64 for the doublet-doublet-triplet comparison. The remaining portions of the sequence, i.e. 15/16 and 57/64 of the sequence is determined, and this allows one to deduce the remaining singlet sequences.

In the simulation of a doublet-doublet-doublet comparison, 78 out of 80 bases could be uniquely assigned for all four singlet sequences. In the doublet-doublet-triplet comparison 73 out of 80 bases could be uniquely assigned for all three singlet sequences. This is far in excess of the 8 bases required to uniquely identify a given singlet sequence.

Sequencing of *DrdI* island PCR fragments from BACs, or directly off BACs.

As discussed *supra*, a method was provided for sequencing DNA directly from the plasmid or cosmid clone by PCR amplification of the insert. While PCR amplification has not been demonstrated for DNA of BAC clone length, the *DrdI* island may be PCR amplified by using a second frequent cutter enzyme to create small fragments for amplification. The second enzyme would contain a two base 5' overhang such that ligation/cutting could proceed in a single reaction tube. The ligation primers/PCR primers can be designed such that *only DrdI*-second enzyme fragments amplify. PCR primers may be removed by using ribose containing primers and destroying them with either base (i.e. 0.1N NaOH) or using dU and UNG. An alternative approach to sequence DNA directly from PCR-amplified DNA uses ultrafiltration in a 96 well format to simply remove primers and dNTPs physically, and is commercially available from Millipore.

Examples of frequent enzymes with TA overhangs (and frequency in the human genome) are: *Bfa*I (CTAG, 1 every 350 bp), Csp6I (GTAC, 1 every 500 bp) and *Mse*I (TTAA, 1 every 133 bp). For fragments with larger average sizes, four base recognition enzymes with CG overhangs may be used: *Msp*I (CCGG, 1 every 2.1 kb), *HinP1*I (GCGC, 1 every 2.5 kb), and *Taq*I (TCGA, 1 every 2.6kb).

There is a chance that the second site enzyme cleaves either too close to a *Drd*I site to generate sufficient sequence or, alternatively, too distantly to amplify efficiently. This site will simply not be scored in the database, just at *Drd*I sites with palindromic overhangs (i.e. AT) are not scored. If it is critical to obtain that precise sequence information, the problem may be addressed by using a different second enzyme. One advantage of using the "CG" site enzymes is that average fragment sizes will be larger and, consequently, will be amenable to generating neighboring sequence information from the second site if needed. This may be helpful for increasing the density of internal sequence information linked to a BAC clone or plasmid/cosmid clone.

Plasmids containing colE1 replication origins (i.e. pBR322, pUC derivatives) are present at high copy number which may be increased to 100's by growing clones for two days or to 1,000's by amplification with chloramphenicol. This should provide sufficient copy number such that it is not necessary to separate plasmid/cosmid DNA from host bacterial chromosomal DNA. On the other hand, BAC clone vectors are based on the F factor origin of replication may be present at copy numbers equal or only slightly higher than the bacterial chromosome. Thus, it will probably be necessary to partially purify BAC clone DNA from bacterial chromosome DNA. The relative advantages and disadvantages of PCR amplification followed by direct sequencing vs. rapid purification of plasmid, cosmid, or BAC clone followed by sequencing need to be determined experimentally.

Alternative enzymes; *Sap*I and *Bgl*I.

There may be regions of the genome which contain less than two readable *Drd*I sequences. One solution to this problem is to use a second enzyme with a comparable frequency in the human genome. By slightly modifying the

procedure, 16 linker/primer sets may be used on split palindrome enzymes which generate a 3 base 3' overhang. Since the overhang is an odd number of bases, it is not necessary to exclude the palindromic two base sequences AT, TA, GC, and CG. To reduce the number of ligations from 64 (all the different possible 3 base overhangs) to 16, the linkers and primers are degenerate at the third position, i.e. end with NTC or NGC. Since there are 3 levels of specificity in the ligation and sequencing step, the third base degeneracy will not interfere with the fidelity of the reaction.

Of the 4 commercially available split palindrome enzymes which generate a 3 base 3' overhang, *Bgl*I (GCCNNNN^NGGC (SEQ. ID. No. 21)) and *Dra*III (CACNNN^GTG) are present at low enough frequencies to be compatible with *Drd*I. There are 60 *Bgl*I sites in about 550 kb of the four sequenced BAC clones, or an average of 1 *Bgl*I site per 9 kb. Since the linkers can ligate to both sides of a *Bgl*I site, there are twice as many ends, (i.e. sequences) generated as with the *Drd*I sites. See Figure 16. Using *Bgl*I, there are two levels of specificity for creating a unique representation: (i) ligation of the top strand, and (ii) extension of the sequencing primer with polymerase. Unlike *Drd*I, the use of a last base degeneracy in the *Bgl*I linker does not allow one to determine sequence information from only one side. If there are too many *Bgl*I sites in a given BAC, or there is a need to obtain singlet sequence information, one may obtain additional specificity by designing primers which reach in one additional base on the 3' side of the ligation junction (i.e. GCCNNNN^NGGC (SEQ. ID. No. 22)). As with *Drd*I, the conserved GGC on the 3' side of the cut site allows all sequences in a set to be easily compared in the correct alignment. As with the *Drd*I site, use of a second enzyme or enzyme pair (*Msp*I and/or *Taq*I) and corresponding linkers allows for specific amplification of the *Bgl*I site fragments (See Figure 16A).

One type IIs enzyme, *Sap*I (GCTCTTCN1/4), generates a 3 base 5' overhang 3' which allows for unidirectional ligation, i.e. simultaneous cutting and ligation will only provide the sequence from one side. See Figure 17. There are 69 *Sap*I sites in about 550 kb of the four sequenced BAC clones, or an average of 1 *Sap*I site per 8 kb. One advantage of *Sap*I is that most vectors lack this site. Two disadvantages of *Sap*I are the 5' 3 base overhang will be filled in if using the enzyme after a PCR amplification, and the need to test a few (5-10) different starting positions

to align doublet or triplet sequences precisely with each other. If there is a need to obtain a singlet sequence, one may obtain additional specificity by designing primers which reach in one or two additional base on the 3' side of the ligation junction (i.e. GCTCTTCN^NNNNN (SEQ. ID. No. 23)). One big advantage of using this enzyme is the majority of *Sap*I sequences yield singlet reads.

The probabilities of obtaining two readable sequencing runs from a BAC clone containing from 2 to 36 *Bgl*I or *Sap*I sites have been calculated. For the average of 12-17 *Bgl*I sites per BAC clone (=24-34 ends), the probability is 99.9% for containing at least two readable (singlet or doublet) sequences. For the same clones, from 93%-98% will contain at least two singlet sequences, making alignment even easier for those clones. For the average of 12-25 *Sap*I sites per BAC clone, the probability is 99.9% for containing at least two readable (singlet or doublet) sequences. For the same clones, from 98.8%-99.3% will contain at least two singlet sequences, making alignment even easier for those clones (see Figure 17A).

Although there are a total of 16 different ligation primers which may be used with the *Bgl*I or *Sap*I sites (indeed, up to 64 may be used), it is not necessary to use all of them. Given the frequency of *Bgl*I sites in the human genome, and the fact that a single site provides two non-symmetric overhangs, 8 different ligation primers would be sufficient. Should a *Bgl*I site be present in low abundance repetitive DNA, that overhang would also not be used. Simulation on a dozen BAC clones would provide a more complete assessment of which 8 primers should be chosen for a *Bgl*I representation. With *Sap*I, each site provides one non-symmetric overhang, so the majority of *Sap*I sites per BAC clone provide singlet or doublet reads. Thus, anywhere from 6 to 10 different ligation primers may be chosen to provide a robust set of *Sap*I islands to assure overlap of all the BAC clones. The advantage of using *Bgl*I or *Sap*I with 6 to 10 different ligation primers is that additional primers may be used as needed on only those BAC clones which represent the ends of contigs. The underlying concept is that each unique linker creates a set of sequences which may be linked through singlet and doublet reads, or BAC clone overlap, or both.

Presence of *DrdI* or other sites in BAC or plasmid vectors.

One important technical note is that the most common BAC vector, pBeloBAC11 (Genbank Accession # U51113 for complete DNA sequence) and the common plasmid vectors contain 4 and 2 *DrdI* sites respectively.

Thus, one needs to fine tune the experimental approach to circumvent restriction sites in the vector sequences. The three basic approaches are to (i) remove the restriction sites from the vector before constructing the library, (ii) destroy the vector restriction sites in clones from a given library, or (iii) suppress amplification of vector fragments using sequence specific clamping primers.

Restriction sites can be removed from the BAC vector pBeloBAC11 which contains 4 *DrdI* sites, 4 *BglI* sites, and 2 *SapI* sites. See Figure 21. The procedure for removing *DrdI* sites in a single cloning step will be described, and it is generally applicable to all the sites. One of the tricks of split palindrome enzymes which generate a 3 base 3' overhang such as *BglI* (GCCNNNN^NGGC (SEQ. ID. No. 21)), *Dra*III (CACNNN^GTG), *AlwnI* (CAGNNN^CTG), and *PflMI* (CCANNNN^NTGG (SEQ. ID. No. 24)) is that there is a high chance of creating fragments where all the sticky ends are unique. In such a case, a plasmid may be cleaved with the enzyme, one or more pieces replaced, and, then, in the presence of T4 ligase, the plasmid reassembles correctly and can be recovered by transforming into *E. coli*. The replacement fragments lack the *DrdI* site(s) such that silent mutation(s) are introduced into any open reading frames. The replacement fragments are generated by overlap PCR, and the ends of such PCR fragments converted to unique overhangs using the split palindrome enzyme (i.e. *BglI*). To illustrate with pBeloBAC11, two overlap PCR primers are designed to eliminate the *DrdI* site at 1,704, and the fragment is generated using two primers just outside *BglI* sites at 634 and 2,533. This fragment is cleaved with *BglI* after PCR amplification. Likewise, six overlap PCR primers are designed to eliminate the *DrdI* sites at 2,616, 3,511, and 4,807 and the whole fragment is generated using two primers just outside *BglI* sites at 2,533 and 6,982. This fragment is also cleaved with *BglI* after PCR amplification. The fragments are mixed with *BglI* cut pBeloBAC11, and ligase is added, in the presence of *DrdI*. Thus, circular ligation products containing the newly PCR

amplified fragments lacking *Drd*I sites are selected for, and recovered after transformation into *E. coli*. The pBeloBAC11 vector has been modified (in collaboration with New England Biolabs) essentially as described above to create vector pBeloBAC11 No *Drd*I, which as its name implies, lacks *Drd*I sites. The same principle may be used to remove the *Sap*I sites and even the *Bgl*I sites or all 10 sites if desired. In the latter case, the split palindrome enzyme *Pfl*MI (4 sites in pBeloBAC11) would be used. The same procedure may be applied to plasmid vectors such as pUC19, which contain only 2 each of *Drd*I and *Bgl*I sites and no *Sap*I sites. See Figure 24.

The vector restriction site or its sequence can be destroyed by treating the vector-insert DNA with various restriction enzymes. The vector sites can be eliminated so that the (*Drd*I) enzyme does not cut at that position or, alternatively, generates such a small sequence (i.e. 10-20 bases) that overlap from vector sequence only minimally interferes with interpretation of the data. This may appear as extra work; however, when using simultaneous restriction/ligation conditions, it is simply a matter of including (an) additional restriction endonuclease(s) in the same mixture. The linker primers will not ligate onto the other restriction site overhangs as they are not compatible.

Representational amplification from BACs may be modified to suppress amplification of vector fragments using sequence specific clamping primers. The pBeloBAC11 and pBACe3.6 vectors both contain *Drd*I sites complementary to AA-, CA-, and GA- overhangs. Clamping oligonucleotides which bind specific *Drd*I fragments (i.e. vector derived) and block annealing of PCR primers or PCR amplification, were designed as PNA or propynyl derivatives and are listed in Tables 5 and 6.

Table 5. PNA designed for suppression of DrdI sites associated with the pBeloBAC11 vector.

Primer	Sequence ($NH_2 \rightarrow CONH_2$)
CA-PNA27-3	NH_2 GCC AGT CGG AGC ATC AGG $CONH_2$ (SEQ. ID. No. 25)
GA-PNA23-4	NH_2 CCC CGT GGA TAA GTG GAT $CONH_2$ (SEQ. ID. No. 26)
GA-PNA25-2	NH_2 ACA CGG CTG CGG CGA GCG $CONH_2$ (SEQ. ID. No. 27)
AA-PNA21	NH_2 GCC GCC GCT GCT GCT GAC $CONH_2$ (SEQ. ID. No. 28)

Table 6. Propynyl Primers designed for suppression of *DrdI* sites associated with the pBeloBAC11 vector.

Primer	Sequence (5'→3')
AA Dcl PY3	5' GsCs(pC) sGsCs(pC) sGCT G(pC)T G(pC)T GA(pC) GG(pT) GTG A(pC)G TT -Bk 3' (SEQ. ID. No. 29)
GA Cl PY6	5' GsAs(pC) sTsGsT s(pC)AT T(pT)G AGG G(pT)G AT(pT) TGT (pC)AC A(pC)T GAA AGG G -Bk 3' (SEQ. ID. No. 30)
GA Cl PY10	5'CsAs(pT) sAsGsT s(pC)TG AGG G(pT)T AT(pC) TGT (pC)AC AGA T(pT)T GAG GG(pT) GG-Bk 3' (SEQ. ID. No. 31)
CA Cl PY14	5' CsAs(pT) sAsGsT s(pC)AT GAG (pC)AA (pC)AG TTT (pC)AA TGG (pC)CA GT(pC) GG - Bk 3' 3' (SEQ. ID. No. 32)

The designations (pC) and (pT) represent propynyl-dC and propynyl-dT, respectively.

The PNA oligonucleotides were designed to maximize Tm values in an 18mer sequence, while attempting to also maximize pyrimidine content and avoiding three purines in a row. The propynyl derivative oligonucleotides were designed to overlap the *DrdI* site by two bases, and to contain a total of about 5 to 9 and preferably 7 propynyl dC and propynyl dU groups to increase the Tm, as well as about 4 to 8 and, preferably, 6 thiophosphate groups at the 5' side to avoid 5'-3' exonuclease digestion by *Taq* polymerase during amplification. (Propynyl derivatives are known to increase oligonucleotide Tm values by approximately 1.5-1.7°C per modification, while thiophosphate modifications slightly reduce Tm values by about 0.5°C per modification). These propynyl derivative clamping oligonucleotides were from approximately 25 to 40 bases in length. Alternative propynyl designs which do not overlap the *DrdI* site would also be predicted to suppress vector amplification. Alternative nucleotide modifications which both increase Tm values and prevent 5'-3' exonuclease digestion by *Taq* polymerase, such as 2'o-methyl derivatives, may also be used. Tm values for both PNA and propynyl derivative clamps were generally

above 85°C and, preferably, above 90°C to achieve effective clamping. When the propynyl derivative clamping oligonucleotides were synthesized without either the propynyl or thiophosphate modifications, they were insufficient to effectively block amplification of vector sequences. In general, reactions using 10 ng of digested/linker ligated BAC DNA were subjected to 30-35 cycles (94°C, 15 sec., 65°C, 2 minutes) of PCR amplifications using 25 picomoles each of primers and 50 picomoles of the corresponding clamp. These conditions were sufficient to allow for amplification of insert *Drd*I representational fragments while inhibiting amplification of the vector sequences. The principles of using PNA clamps to suppress amplification of undesired fragments have been described in the literature (Cochet O. et. al. "Selective PCR Amplification of Functional Immunoglobulin Light Chain from Hybridoma Containing the Aberrant MOPC 21-Derived V kappa by PNA-mediated PCR Clamping," Biotechniques 26:818-822 (1999) and Kyger E. et. al. "Detection of the Hereditary Hemochromatosis Gene Mutation by Real-time Fluorescence Polymerase Chain Reaction and Peptide Nucleic Acid Clamping," Anal Biochem 260:142-148 (1998), which are hereby incorporated by reference).

IV. Comparison of *Drd*I Island Approach With Other Endonucleases

Different approaches to generate representations of the genome.

The *Drd*I is a unique restriction endonuclease. It has an infrequent 6 base recognition sequence and generates a degenerate 2 base 3' overhang (GACNNNN^NNGTC). Sequences adjacent to a *Drd*I site may be PCR amplified using the 2 degenerate bases in the overhang to define a representation, and an adjacent more common site (such as *Msp*I). The degenerate 2 base 3' overhang allows for both biochemical selection and bubble PCR to assure that only the *Drd*I island amplifies (and not the more abundant *Msp*I – *Msp*I fragments). Using *Drd*I, there are three levels of specificity for creating a unique representation: (i) ligation of the top strand, (ii) ligation of the bottom strand linker, and (iii) extension of the sequencing primer with polymerase. In addition, if there are too many *Drd*I sites in a given BAC clone, or there is a need to obtain singlet sequence information, one may obtain additional specificity by designing primers which reach in one or two

additional bases on the 3' side of the ligation junction (i.e. GACNNNN^NNGTC (SEQ. ID. No. 33)), since the central degenerate bases are determined by the specificity of the ligation reaction (i.e. GACNNNN^NNGTC (SEQ. ID. No. 33)). Further, the conserved GTC on the 3' side of the cut site allows all sequences in a set to be easily compared in the correct alignment. Finally, the degenerate 2 base overhang allows one to obtain sequence information from either one, or the other, or both sides of the *Drd*I site.

However, there may be a need to consider other restriction endonuclease sites, for example, when starting with a library made from a BAC vector with too many *Drd*I sites.

The use of split palindromic enzymes which generate a 3 base 3' overhang, such as *Bgl*I (GCCNNNN^NGGC (SEQ. ID. No. 21)) and type IIs enzyme, like *Sap*I (GCTCTTCN1/4), which generates a 3 base 5' overhang are described above.

A seemingly simple solution to obtaining sequence information is to use a symmetric palindromic enzyme, such as *Bam*HI, which cuts the BAC at several places. Figure 25 is a schematic drawing showing the sequencing of *Bam*HI islands in random BAC clones in accordance with the present invention. This procedure is largely the same as was described previously for *Drd*I, *Bgl*I, and *Sap*I islands with respect to Figures 1, 5, 16, and 17. After linker ligation, some of the fragments will be under 4 kb and, thus, will amplify in a PCR reaction. The idea here is to amplify all the fragments in a single tube and, then, obtain a representation through use of carefully designed sequencing primers. The selectivity in this type of representation is achieved by using a sequencing primer, whose last two bases extend beyond the *Bam*HI site (i.e. G^GATTCNN). It would be difficult to achieve a specificity of 3 bases beyond the site. In the example of the 170 kb BAC containing the Met Oncogene, there was considerable clustering of the sites which were close enough to amplify effectively. The results of using *Bam*HI as the restriction enzyme are shown in Figure 26.

It is also difficult to find an enzyme which cleaves the DNA frequently enough that some fragments are under 4kb, but not so frequent that too many

fragments amplify, as when using *Eco*RI or *Hin*dIII. Use of enzymes which are less frequent due to a TAG stop codon in one of the potential reading frames (*Avr*II, C^CTAGG; *Nhe*I, G^CTAGC, and *Spe*I A^CTAGT) also have problems with clustering. The results of using these enzymes as the restriction enzyme in accordance with the present invention are shown in Figure 27.

Other symmetric palindromic enzymes which may be used are: *Kpn*I, *Sph*I, *Aat*II, *Age*I, *Xma*I, *Ngo*MI, *Bsp*EI, *Mlu*I, *Sac*II, *Bsi*WI, *Pst*I, and *Apa*LI.

To overcome the above clustering problem, one could use an enzyme which cuts more frequently due to a degeneracy, but then use linkers with only one of the 2 or 4 possible degeneracies such that only a few fragments amplify. For example, *Acc*I has 4 different recognition sequences (GT^MKAC = GT^ATAC, GT^AGAC, GT^CTAC, and GT^CGAC), and *Bsi*HKAI also has 4 different recognition sequences (GWGCW^C = GAGCA^C, GAGCT^C, GTGCA^C, and GTGCT^C). Again, the selectivity in this type of representation is achieved by using a sequencing primer, whose last two bases extend beyond the *Bsi*HKAI site (i.e. GAGCA^CNN). The advantage of these types of restriction sites is that a non-palindromic overhang may be used for the linker. In simulations of these sites on the 171 kb BAC, only a few fragments amplify, including some which would provide too few bases of sequence information to be meaningful (i.e. 19-44 bp). Figure 28 is a schematic drawing showing the sequencing of *Bsi*HKAI islands in random BAC clones in accordance with the present invention. This procedure is largely the same as was described previously for *Drd*I, *Bgl*I, and *Sap*I islands with respect to Figures 1, 5, 16, and 17. The results of using *Bsi*HKAI and *Acc*I as the restriction enzymes are shown in Figure 29.

An alternative is to use an infrequent restriction endonuclease site with a middle base degeneracy in combination with a more frequent cutter, analogous to use of *Drd*I as described earlier. By using a primer for only one of the degenerate sequences, one can obtain sequence information from either one or the other side of the site, such as by using *San*DI (GG^GWCCC). Here, however, all the fragments are amplified simultaneously in the initial PCR, and selectivity is achieved by using a sequencing primer, whose last two bases extend beyond the recognition site (GG^GWCCCNN). Another site, *Sex*AI (A^CCWGGT), may also work, however,

the 5 base overhang may be large enough to allow substantial misligations of primer to overhangs containing a mismatch. In simulations on the 171 kb BAC, all *San*DI and *Sex*AI sites were singlet or doublet reads. Figure 30 is a schematic drawing showing the sequencing of *San*DI islands in random BAC clones in accordance with the present invention. This procedure is largely the same as was described previously for *Drd*I, *Bgl*I, and *Sap*I islands with respect to Figures 1, 5, 16, and 17. The results of using *San*DI and *Sex*AI as restriction enzymes are shown in Figure 31.

*Rsr*II (CG^GWCCG) is an enzyme which provides the same overhang, but is found less frequently than *San*DI. For cases where a higher frequency site is required, the enzymes *Ppu*I (RG^GWCCY), *Ava*II (G^GWCC), *Eco*O109 (RG^GNCCY), or *Bsu*36I (CC^TNAGG) may be used.

Presence of *Drd*I or other sites in BAC or plasmid vectors.

One important technical note is that the most common BAC vector, pBeloBAC11 contains 4 *Drd*I sites, 4 *Bgl*I sites, 2 *Sap*I sites, 6 *Acc*I sites, 8 *Bsi*HKAI sites, 1 *Spe*I site, 1 *Bam*HI site, and 1 *Sex*AI site. See Figures 23 and 32-34.

As discussed above, there are three basic approaches to circumvent the problem of the cloning vector having its own restriction sites: (i) remove the restriction sites from the vector before constructing the library, (ii) destroy the vector restriction sites in clones from a given library, or (iii) ignore the vector restriction sites and use more selective sequencing primers. For the sites described above, the *Acc*I, *Bsi*HKAI, *Spe*I, and *Bam*HI sites do not require additional modification of the pBeloBAC11 vector, because the amplification strategy with these sites need two neighboring sites of the correct sequence to create a PCR fragment. In addition, pBeloBAC11 does not contain any *Avr*II, *Nhe*I, or *San*DI sites.

Distribution of representative *Drd*I and *San*DI sites in the genome.

A number of advanced BLAST searches of the current dbest and dbsts databases were performed to determine if there are any unanticipated biases in the distribution of *Drd*I and in a smaller survey of *San*DI sites.

Distribution of representative *DrdI* sites in the genome.

1. Query: GACAAAANNGTC (SEQ. ID. No. 34)

Expect 100
Filter: None
Other Advanced Options: M=1 N=-4 S=12 S2=12

Non-redundant DBEST Division 1,814,938 sequences; 685,416,569 total letters.
DBSTS Division 59,288 sequences; 21,143,395 total letters.

Query:	1 GACAAAAAAGTC 12	dbest 51	dbsts 3
Query:	1 GACAAAAACGTC 12	dbest 20	dbsts (0)
Query:	1 GACAAAAAGGTC 12	dbest 28	dbsts 1
Query:	1 GACAAAAATGTC 12	dbest 77	dbsts 4
Query:	1 GACAAAACAGTC 12	dbest 86	dbsts (0)
Query:	1 GACAAAACCGTC 12	dbest 5	dbsts (0)
Query:	1 GACAAAACGGTC 12	dbest 4	dbsts (0)
Query:	1 GACAAAACTGTC 12	dbest 96	dbsts 3
Query:	1 GACAAAAGAGTC 12	dbest 62	dbsts 1
Query:	1 GACAAAAGCGTC 12	dbest 6	dbsts (0)
Query:	1 GACAAAAGGGTC 12	dbest 20	dbsts 4
Query:	1 GACAAAAGTGTC 12	dbest 89	dbsts 1
Query:	1 GACAAAATAGTC 12	dbest 9	dbsts 4
Query:	1 GACAAAATCGTC 12	dbest 4	dbsts 1
Query:	1 GACAAAATGGTC 12	dbest 29	dbsts (0)
Query:	1 GACAAAATTGTC 12	dbest 45	dbsts 2
Total =		633	24

2. Query: GACAAACNNGTC (SEQ. ID. No. 35)

Expect 100
Filter: None
Other Advanced Options: M=1 N=-4 S=12 S2=12

Non-redundant DBEST Division 1,814,938 sequences; 685,416,569 total letters.
DBSTS Division 59,288 sequences; 21,143,395 total letters.

Query:	1 GACAAACAAGTC 12	dbest 49	dbsts 2	
Query:	1 GACAAACACGTC 12	dbest 47	dbsts 2	
Query:	1 GACAAACAGGTC 12	dbest 20	dbsts 5	
Query:	1 GACAAACAGGTC 12	dbest 22	dbsts 5	
Query:	1 GACAAACCAGTC 12	dbest 29	dbsts 1	
Query:	1 GACAAACCCGTC 12	dbest 14	dbsts 1	
Query:	1 GACAAACCGGTC 12	dbest 3	dbsts (0)	
Query:	1 GACAAACCTGTC 12	dbest 17	dbsts 3	
Query:	1 GACAAACGAGTC 12	dbest 21	dbsts (0)	
Query:	1 GACAAACGCGTC 12	dbest 15	dbsts 1	
Query:	1 GACAAACGGGTC 12	dbest 8	dbsts (0)	
Query:	1 GACAAACGTGTC 12	dbest 33	dbsts 7	
Query:	1 GACAAACTAGTC 12	dbest 15	dbsts 1	
Query:	1 GACAAACTCGTC 12	dbest 8	dbsts (0)	
Query:	1 GACAAACTGGTC 12	dbest 40	dbsts 2	
Query:	1 GACAAACTTGTC 12	dbest 59	dbsts 2	
Total =		400	32	

3. Query: GACAAAGNNGTC (SEQ. ID. No. 36)

Expect 100
Filter: None
Other Advanced Options: M=1 N=-4 S=12 S2=12

Non-redundant DBEST Division 1,814,938 sequences; 685,416,569 total letters.
DBSTS Division 59,288 sequences; 21,143,395 total letters.

Query:	1 GACAAAGAAGTC 12	dbest 43	dbsts 0	
Query:	1 GACAAAGACGTC 12	dbest 6	dbsts 1	
Query:	1 GACAAAGAGGTC 12	dbest 62	dbsts 2	
Query:	1 GACAAAGATGTC 12	dbest 29	dbsts 5	
Query:	1 GACAAAGCAGTC 12	dbest 31	dbsts 3	
Query:	1 GACAAAGCCGTC 12	dbest 49	dbsts (0)	
Query:	1 GACAAAGCGGTC 12	dbest 5	dbsts (0)	
Query:	1 GACAAAGCTGTC 12	dbest 5	dbsts 1	
Query:	1 GACAAAGGAGTC 12	dbest 15	dbsts 1	
Query:	1 GACAAAGGCGTC 12	dbest 8	dbsts 1	
Query:	1 GACAAAGGGGTC 12	dbest 36	dbsts (0)	
Query:	1 GACAAAGGTGTC 12	dbest 14	dbsts (0)	
Query:	1 GACAAAGTAGTC 12	dbest 7	dbsts (0)	
Query:	1 GACAAAGTCGTC 12	dbest 21	dbsts (0)	
Query:	1 GACAAAGTGGTC 12	dbest 94	dbsts 4	
Query:	1 GACAAAGTTGTC 12	dbest 21	dbsts (0)	
Total =		446	18	

4. Query: TCTGGGACCCNN (SEQ. ID. No. 37)

```
Expect 100
Filter:  None
Other Advanced Options:  M=1 N=-4 S=12 S2=12

Database:  Non-redundant Database of GenBank STS Division
           59,293 sequences; 21,148,385 total letters.

                                          Dbsts

Query:     1 TCTGGGACCCAA 12              3

Query:     1 TCTGGGACCCAC 12              1

Query:     1 TCTGGGACCCAG 12              7

Query:     1 TCTGGGACCCAT 12              2


Query:     1 TCTGGGACCCCA 12              6

Query:     1 TCTGGGACCCCC 12              6

Query:     1 TCTGGGACCCCG 12              1

Query:     1 TCTGGGACCCCT 12              5


Query:     1 TCTGGGACCCGA 12             (0)

Query:     1 TCTGGGACCCGC 12              1

Query:     1 TCTGGGACCCGG 12              3

Query:     1 TCTGGGACCCGT 12             (0)


Query:     1 TCTGGGACCCTA 12              2

Query:     1 TCTGGGACCCTC 12              8

Query:     1 TCTGGGACCCTG 12              3

Query:     1 TCTGGGACCCTT 12              5


Total                                    53
```

The advanced BLAST search requires a minimum of 12 bases to look for an exact match. In the initial stages of doing this search, the database computer

went down (probably unrelated); however, as a precaution, responses for a particular sequence search were limited to 100. Since the dbest database contains about 1/4 nonhuman sequence, such sequences were removed in tallying the total for that search. Thus, any number between 75 and 100 most probably reflects a lower value for that particular *DrdI* site. Nevertheless, since many dbest searches returned less than 100 hits, it is unlikely that a particular total is grossly under-represented. Nevertheless, to be accurate, the following values should be viewed as lower estimates.

For the *DrdI* site, there are 6 non-palindromic two base 3' overhangs to consider: AA, AC, AG, CA, GA, and GG. Searches were performed on a representation of AA, AC, and AG sequences. The first two bases in the middle N6 degenerate sequence were arbitrarily chosen as "AA", the next two bases were AA, AC, or AG, and the last two bases were entered 16 times for each of the NN possibilities.

For all three searches (i.e., GACAAAAANNGTC (SEQ. ID. No. 34), GACAAACNNGTC (SEQ. ID. No. 35), and GACAAAGNNGTC (SEQ. ID. No. 36)), sequences containing a CG dinucleotide in either database or a "TAG" trinucleotide in the dbest database were, as expected, underrepresented. The STS database is too small to draw major conclusions; however, the totals on all three searches were within 2-fold of each other.

For the STS database of less than 21,000,000, 18 - 32 hits of human sequence were obtained which correlates to 1 site in 700,000 – 1,100,000 bases.

For the dbest database of less than 685,000,000, 400 - 633 hits of human sequence were obtained which correlates to 1 site in 1,100,000 to 1,700,000 bases.

Again, the middle N6 has 4096 different sequences. Because of the palindromic nature of GACAAAAAAGTC (SEQ. ID. No. 38), whenever it was searched, the program automatically also searched GACTTTTTTGTC (SEQ. ID. No. 39), and each middle AA sequence was searched with 16 different flanking dinucleotides. All the sequences with a middle AA or TT is 4096/8 = 512, then divide by 16 = 32.

For the best results, 400, 446, and 633 sequences in 685,000,000 is equivalent to 1,752, 1,953, and 2,772 sequences, respectively, in 3,000,000,000. It should be a little more, because the 685,000,000 contains approximately 1/4 sequence which is non-human DNA.

So the total number of *Drd*I sites with AC, AG, and AA overhangs are 32 x 1,752; 1,953; and 2,772; = 56,064; 62,496; and 88,704 sites, respectively. Since A-T bases are somewhat more frequent in the genome than G-C bases, the above numbers are a slight over-representation. This occurs, because they are based on numbers obtained using "AA" as the arbitrarily chosen invariant first two bases in the *Drd*I internal sequence. For the other 3 middle 2 base overhangs, "CA" is predicted to be as frequent as "AG", i.e. about 60,000 sites; "GA" (whose complement is "TC") is predicted to be as frequent as "AC", i.e. about 55,000 sites; and "GG" (whose complement is "CC") is predicted to be less frequent than "AC", i.e. about 45,000 sites.

The above calculations are consistent with the earlier prediction of 200,000 to 300,000 non-palindromic *Drd*I sites per genome; i.e. on average of 33,000 to 50,000 sites for each overhang.

Less detailed searches with *San*DI were performed by arbitrarily choosing the first 3 bases of a 12 base sequence as "TCT" and using the GGGACCC site with the last two bases being entered 16 times for each of the NN possibilities.

For the STS database of less than 21,000,000, 53 hits of human sequence were obtained which equals 1 site in 400,000 bases. 53 in 21,000,000 is equivalent to 7,571 in 3,000,000,000. Since there are 64 different combinations for the first 3 bases, that gives a prediction of 484,571 *San*DI sites in the genome. These may be divided into 16 sets, on average of 30,000 sites per set.

The database searches demonstrate the distribution of *Drd*I sites (as well as *San*DI and other selected sites) allow for the creation of from 5 to 16 sets based on specific 2 base overhangs or neighboring 2 bases, where each set has from about 30,000 to about 90,000 members, and may be used to create entire genome overlapping contig maps.

Option 1: 1,800,000 short sequencing reactions generate approximately 100,000-150,000 *DrdI* islands to create an entire BAC contig.

Figure 2 provides a scheme for sequencing representations of BAC clones. Two approaches may be considered for preparing DNA. One rapid approach is to pick individual colonies into lysis buffer and lyse cells under conditions which fragment chromosomal DNA but leave BAC DNA intact. Chromosomal DNA is digested by the ATP dependent DNase from Epicentre which leaves CCC and OC BAC DNA intact. After heat treatment to inactivate the DNase, restriction digestion, ligation of linker adapters, and PCR amplification are all performed in a single tube. The products are then aliquoted and sequencing is performed using specific primers to the adapters. This first approach has the advantage of obviating the need to grow and store 300,000 BAC clones.

An alternative approach is to pick the colonies into 1.2 ml growth media and make a replica into fresh media for storage before pelleting and preparing crude BAC DNA from a given liquid culture similar as described above. This second approach has the advantage of producing more BAC DNA, such that loss of an island from PCR dropout is less likely. Further, this approach keeps a biological record of all the BACs, which may become useful in the future for techniques such as exon trapping, transfection into cells, or methods as yet undeveloped.

Figures 5 is an expanded versions of Figure 2 detailing the subtleties of the linker-adapter ligations and bubble PCR amplification to select only the *DrdI*-*MspI* fragments. Figure 7 describes the three levels of specificity in using the *DrdI* island approach.

With an average BAC size of 100-150 kb, total of 20,000 to 30,000 BAC clones would cover the human genome, or 300,000 clones would provide at least 10-fold coverage. For each clone, one requires 6 sequencing runs for a total of 1,800,000 sequencing reactions. However, only 80 bp of sequence is required to deconvolute singlet/doublet information. At a conservative estimate of 1 run per hour of 96 reaction, with 24 loadings/day, this equals 2,304 sequencing reads/PE 3700 machine/day. Assume access to 200 machines.

1,800,000/2,304 sequencing reads/machine/day = 885 machines days/200 machines = 4.4 days

The above would provide about 80 bp anchored sequence information for about 100,000 to 150,000 *DrdI* sites, spaced on average every 20-30 kb.

If the machine is run to read 200-300 bp, this equals 1,240 reads/day, then:

1,800,000/1,240 sequencing reads/machine/day = 1,452 machines days/200 machines = 7.3 days

The above would provide about 200-300 bp anchored sequence information for about 100,000 to 150,000 *DrdI* sites, spaced on average every 20-30 kb.

If the machine is run to read 500-600 bp, this equals 760 reads/day, then:

1,800,000/760 sequencing reads/machine/day = 2,368 machines days/200 machines = 11.8 days

The above would provide about 500-600 bp anchored sequence information for about 100,000 to 150,000 *DrdI* sites, spaced on average every 20-30 kb.

Experiments will be needed to access the quality of reads and ability to deconvolute the sequence when reading out 80, 200, or 500 bp. In simulations, it was noted that doublets often contained one smaller and one larger fragment. Thus, useful information may be obtained from a long read, where the first 200 bases are predominantly from the shorter fragment (reading as a strong singlet sequence with a weak doublet behind it), and when that fragment ends, the weaker sequence from the larger fragment will be easy to read and interpret (See Figure 35). This may require the algorithm to include alignment of fragments starting at a later position; however, this should not be too difficult.

Option 2: 3,6 00,000 short sequencing reactions generate approximately 150,000-200,000 *Drd*I islands to create an entire BAC contig.

Should pilot studies suggest that some sequence reads are difficult to interpret, two sets of *Drd*I islands can be generated for each BAC clone, one set consisting of AA, AC, AG, CA, GA, or GG overhangs, while the other set consists of TT, GT, CT, TG, TC, or CC overhangs. Although most sequences would be represented in both sets, each would rescue *Drd*I islands lost from the other set due to either the neighboring *Taq*I or *Msp*I site being too close (resulting in amplification of a very short fragment which lacks the number of bases required to determine uniqueness) or too far (resulting in weak or no amplification of the longer fragment). In such a circumstance, the number of sequencing runs would double, but the number of useable sequences for alignments would also increase. For the example of the Met oncogene containing BAC on 7q31, the first six linker set provides 3 singlet and 3 doublet sequences. The second six linker set provides an additional 2 singlet and 3 doublet sequences (See Figure 35). Using this very conservative approach, 3,600,000 sequencing runs would be required:

3,600,000/2,304 sequencing reads/machine/day = 1,770 machines days/200 machines = 8.8 days

The above would provide about 80 bp of nchored sequence information for about 150,000 to 200,000 *Drd*I sites, spaced on average every 15-20 kb.

If the machine is run to read 200-300 bp, this equals 1,240 reads/day, then:

3,600,000/1,240 sequencing reads/machine/day = 2,904 machines.days/200 machines = 14.6 days

The above would provide about 200-300 bp anchored sequence information for about 150,000 to 200,000 *Drd*I sites, spaced on average every 15-20 kb.

If the machine is run to read 500-600 bp, this equals 760 reads/day, then:

3,600,000/760 sequencing reads/machine/day = 4,736 machines days/200 machines = 23.6 days

Add to this sequencing, both ends of the 300,000 BAC clones (using unique primers to the two ends and bubble PCR) = 600,000/760 sequencing reads/machine/day = 790 machines days/200 machines = 3.9 days

The above would provide about 500-600 bp anchored sequence information for about 150,000 to 200,000 *Drd*I sites, spaced on average every 15-20 kb. This is approximately 75 million to 120 million anchored bases and is from a 2.5% to 4% representation of the genome. With a 10-fold coverage, and reasonably clean reads, one should be able to identify about 100,000 to 170,000 anchored SNPs in 23.6 days. Further, the ends of the BAC clones will, providing sequencing reads of average length 325 bases for about 75% of the end, for an additional 145 million bases. The BAC end sequences are not completely anchored since one cannot determine orientation of the ends with respect to other BAC clones unless the end sequence fortuitously overlaps with another end sequence in the opposite orientation (predicted to occur 325/150,000 bp = 0.2% of the clones.) Nevertheless, the BAC end sequences are relatively anchored and will provide confirming sequence information once the random sequence from 10 kb insert clones are collected. The total of 28 days sequencing will provide 7.5 to 9% of anchored and relatively anchored genomic sequence.

Alternatively, one can create *Drd*I libraries of 5-pooled individuals DNA in pUC vectors to find the SNPs. As described previously, a size-selection of fragments between 200 and 1,000 bp will provide a 0.26% representation of the genome (average size of 580 bp; number of fragments is 19,700) for a single overhang. If the latter number is multiplied by 12 different overhangs, a 10-fold

coverage is provided, and both strands are sequenced, 20,000 x 12 x 10 = 2,400,000 sequencing runs are obtained.

2,400,000/760 sequencing reads/machine/day = 3,158 machines days/200 machines = 15.8 days

Thus, if the initial reads from the BAC libraries are exceptionally clean, then long reads of 500-600 bp may be used to create an anchored representation with 100,000 to 170,000 SNPs, and can be completed in 23.6 + 3.9 = 27.5 days. Alternatively, much shorter runs may be used for the initial BAC sequencing, and, then, higher quality runs may be used to extend the anchors and create a 200,000 SNP library in 8.8 + 15.6 + 3.9 = 28.3 days.

Option 3: 2,400,000 short sequencing reactions generate approximately 150,000-200,000 *BglI* islands to create an entire BAC contig.

One concept is to increase the number of anchored sites in a given BAC. The *BglI* restriction endonuclease generates a 3 base 3' overhang, but may also be used to create a representation (See Figure 14). Since the overhang is an odd number of bases, it is not necessary to exclude the palindromic two base sequences AT, TA, GC, and CG. To reduce the number of ligations from 64 (all the different possible 3 base overhangs) to 16, the linkers and primers are degenerate at the last position, i.e. end with a 3' ACN or AAN. (Please note: Greater specificity may be achieved by using the degeneracy at the 3' end of the linker adapter.) Since there are 3 levels of specificity in the ligation and sequencing step (see Figure 36), the third base degeneracy will not interfere with the fidelity of the reaction.

Again, with an average BAC size of 100-150 kb, a total of 20,000 to 30,000 BAC clones would cover the human genome, or 300,000 clones would provide at least 10-fold coverage. For each clone, one requires 8 sequencing runs for a total of 2,400,000 sequencing reactions. Using the same assumptions as above:

2,400,000/2,304 sequencing reads/machine/day = 1042 machines days/200 machines = 5.2 days

The above would provide about 80 bp anchored sequence information for about 150,000 to 200,000 *BglI* sites, spaced on average every 15-20 kb.

If the machine is run to read 200-300 bp, this equals 1,240 reads/day, then:

2,400,000/1,240 sequencing reads/machine/day = 1,935 machines days/200 machines = 9.7 days

The above would provide about 200-300 bp anchored sequence information for about 150,000 to 200,000 *BglI* sites, spaced on average every 15-20 kb.

If the machine is run to read 500-600 bp, this equals 760 reads/day, then:

2,400,000/760 sequencing reads/machine/day = 3,158 machines days/200 machines = 15.8 days

The above would provide about 500-600 bp anchored sequence information for about 150,000 to 200,000 *BglI* sites, spaced on average every 15-20 kb.

Option 4: 4,800,000 short sequencing reactions generate approximately 200,000-250,000 *BglI* islands to create an entire BAC contig.

Should pilot studies suggest that some sequence reads are difficult to interpret, two sets of *BglI* islands can be generated for each BAC clone, one set consisting of **AAN**, **CAN**, **GAN**, **TAN**, **AGN**, **CGN**, **GGN**, or **TGN** overhangs, while the other set consists of **ACN**, **CCN**, **GCN**, **TCN**, **ATN**, **CTN**, **GTN**, or **TTN** overhangs. While most sequences would be represented in both sets, each would rescue *BglI* islands lost from the other set due to either the neighboring *TaqI* or *MspI* site being too close (resulting in amplification of a very short fragment which lacks the number of bases required to determine uniqueness) or too far (resulting in weak or

no amplification of the longer fragment). In such a circumstance, the number of sequencing runs would double, but the number of useable sequences for alignments would also increase. For the example of the Met oncogene containing BAC on 7q31, the first eight linker set provides 5 singlet and 3 doublet sequences. The second eight linker set provides an additional 3 doublet sequences (See Figure 35). The set of non-palindromic linker adapters may be mixed, as long as the complement is not also included in the mixer. For example, to chose sites which will allow the PCR primers to end in only a C or A, the set of **A**AN, **C**AN, **G**AN, **T**AN, **A**CN, **C**CN, **G**CN, and **T**CN overhangs may be used (See Figure 35). This set allows design of PCR primers with 3' bases of either "A" or "C", which tend to give less miss-priming than primers with 3' "G" or "T", which may give false PCR amplification products resulting from polymerase extension of a T:G mismatched base. In this BAC, the TGT or ACA overhang appeared too frequently, suggesting it may be associated with a repetitive element. For the purposes of these calculations, the complete set of 16 linkers would require 4,800,000 sequencing runs, although less linkers would most probably suffice:

4,800,000/2,304 sequencing reads/machine/day = 2083 machines days/200 machines = 10.4 days

The above would provide about 80 bp anchored sequence information for about 200,000 to 250,000 *BglI* sites, spaced on average every 12-15 kb.

If the machine is run to read 200-300 bp, this equals 1,240 reads/day, then:

4,800,000/1,240 sequencing reads/machine/day = 3,871 machines days/200 machines = 19.4 days

The above would provide about 200-300 bp anchored sequence information for about 200,000 to 250,000 *BglI* sites, spaced on average every 12-15 kb.

If the machine is run to read 500-600 bp, this equals 760 reads/day, then:

4,800,000/760 sequencing reads/machine/day = 6,316 machines days/200 machines = 31.6 days

The above would provide about 500-600 bp anchored sequence information for about 200,000 to 250,000 *BglI* sites, spaced on average every 12-15 kb.

Add to this sequencing both ends of the 300,000 BAC clones (using unique primers to the two ends and bubble PCR) = 600,000/760 sequencing reads/machine/day = 790 machines days/200 machines = 3.9 days

The above would provide about 500-600 bp anchored sequence information for about 200,000 to 250,000 *BglI* sites, spaced on average every 12-15 kb. This is approximately 100 million to 150 million anchored bases and is from a 3% to 5% representation of the genome. With a 10-fold coverage, and reasonably clean reads, one should be able to identify about 130,000 to 200,000 anchored SNPs in 31.6 days. Further, the ends of the BAC clones will provide an additional 145 million bases of relatively anchored sequences. The total of 36 days sequencing will provide 8 to 10% of anchored and relatively anchored genomic sequence.

As described above, one can create *BglI* libraries of 5-pooled individuals DNA in pUC vectors to find the SNPs. A size-selection of fragments between 200 and 1,000 bp will provide a 0.26% representation of the genome for a single overhang (about 20,000 fragments). If the latter number is multiplied by 16 different overhangs, a 10-fold coverage is provided, and both strands are sequenced, there are 20,000 x 16 x 10 = 3,200,000 sequencing runs.

3,200,000/760 sequencing reads/machine/day = 4,210 machines days/200 machines = 21.0 days

Thus, if the initial reads from the BAC libraries are exceptionally clean, then long reads of 500-600 bp may be used to create an anchored representation with 130,000 to 200,000 SNPs, and can be completed in 31.6 + 3.9 = 35.5 days. Alternatively, much shorter runs may be used for the initial BAC sequencing, and

then higher quality runs may be used to extend the anchors and create a 250,000 SNP library in 10.4 + 21.0 + 3.9 = 35.3 days.

Option 5: 4,200,000 short sequencing reactions generate approximately 250,000-300,000 *DrdI* and *BglI* islands to create an entire BAC contig.

An alternative strategy is to combine the best of both representations, using 6 non-palindromic linker-adapters for the *DrdI* overhangs, and 8 non-palindromic linker-adapters for the *BglI* overhangs (see Figure 37.) If the multiplex PCR of 14 different linker-adapter sets does not amplify all fragments in sufficient yield, then the BAC DNA may be aliquoted initially into two or more tubes. Further, unique primer sets may be used to increase yield of a PCR fragment prior to the sequencing reaction. The advantages of such a hybrid representation is that it maximizes the distribution of independent sequence elements. As noted above, should any *DrdI* or *BglI* site be frequently found in repetitive elements, that overhang can be removed from the representation. For the full representation, the hybrid approach uses 6 + 8 = 14 sequencing runs for each BAC:

4,200,000/2,304 sequencing reads/machine/day = 1,823 machines days/200 machines = 9.1 days

The above would provide about 80 bp anchored sequence information for about 250,000 to 350,000 *DrdI* and *BglI* sites, spaced on average every 8-12 kb.

If the machine is run to read 200-300 bp, this equals 1,240 reads/day, then:

4,200,000/1,240 sequencing reads/machine/day = 3,387 machines days/200 machines = 16.9 days

The above would provide about 200-300 bp anchored sequence information for about 250,000 to 350,000 *DrdI* and *BglI* sites, spaced on average every 8-12 kb.

If the machine is run to read 500-600 bp, this equals 760 reads/day, then:

4,200,000/760 sequencing reads/machine/day = 5,526 machines days/200 machines = 27.6 days

The above would provide about 500-600 bp anchored sequence information for about 250,000 to 350,000 *DrdI* and *BglI* sites, spaced on average every 8-12 kb. This is approximately 125 million to 210 million anchored bases and is from a 4.2% to 7% representation of the genome. With a 10-fold coverage, and reasonably clean reads, one should be able to identify about 180,000 to 300,000 anchored SNPs in 31.6 days. Further, the ends of the BAC clones will provide an additional 145 million bases of relatively anchored sequences. The total of 32 days sequencing will provide 9.2 to 12% of anchored and relatively anchored genomic sequence.

As described above, one can create *BglI* libraries of 5-pooled individuals' DNA in pUC vectors to find the SNPs. A size-selection of fragments between 200 and 1,000 bp will provide a 0.26% representation of the genome for a single overhang (about 20,000 fragments). If the latter number is multiplied by 16 different overhangs, a 10-fold coverage is provided, and both strands are sequenced, 20,000 x 14 x 10 = 2,800,000 sequencing runs are obtained.

2,800,000/760 sequencing reads/machine/day = 3,684 machines days/200 machines = 18.4 days

Thus, if the initial reads from the BAC libraries are exceptionally clean, then long reads of 500-600 bp may be used to create an anchored representation with 180,000 to 300,000 SNPs, and can be completed in 27.6 + 3.9 = 31.5 days. Alternatively, much shorter runs may be used for the initial BAC sequencing, and then higher quality runs may be used to extend the anchors and create a 240,000 SNP library in 9.1 + 18.4 + 3.9 = 31.4 days. In summary, a month and a day of sequencing on 200 machines will provide a valuable database containing anchored

and mapped sequence islands of 500-600 bases on average every 8-12 kb with approximately 240,000 mapped SNP's.

IV. Creating a *DrdI* Island Database of Mapped SNPs and Using a Universal DNA Array for High Throughput Detection of SNPs.

Use of the *DrdI* Island Approach for Alignment of Plural Clones

Figures 38 to 45 show how the *DrdI* island approach of the present invention can be utilized to align 4 hypothetical BAC clones containing 8 to 12 non-palindromic *DrdI* sites. In this example, the 6 linkers with the Group II dinucleotide overhangs (i.e. AG, AC, CA, GA, AA, and GG) are used. The *DrdI* sites are labeled 1a, 1b, 1c ...., 2a, 2b, ... up to 6a, 6b, ... The numeral represents the type of non-palindromic 2 base overhang for that *DrdI* site: 1 = AA, 2 = AC, 3 = AG, 4 = CA, 5 = GA, and 6 = GG. The lower-case letter represents the first = a, second = b, third = c, and so on, for each unique sequence with that particular non-palindromic 2 base overhang. As described more fully below, each of the 6 linkers generates a separate representation of overlapping islands on the 4 different BAC clones. When the different representations obtained with each linker in the *DrdI* island analysis are combined, the alignment of the BAC clones can be determined.

In each of Figures 38-44, the top panel illustrates the actual position of each *DrdI* site within each BAC, the *DrdI* island data generated from each of these BAC clones is provided in the table below. After obtaining sequence information in each clone, one compares the sequences in each column and determines if the two entries are concordant or discordant as described *supra*. The BAC clones overlap if the entries in that column are concordant. The BAC clones do not overlap if all the entries in all the columns are discordant. Since a large scale sequencing project will produce from about 30,000 to 90,000 entries in each column, virtually all the clones will be discordant with each other, only a few will overlap with each other at a given point in the contig. The number of different ways to establish overlap between two BAC clones is considerable.

In Figure 38, the *DrdI* island approach is used to determine sites with AA overhangs. When the procedure described *supra* with respect to Figure 1 is

carried out, for AA overhangs, BAC clone I is found to have a triplet, BAC clone II has a doublet, BAC clone III has a doublet, and BAC clone IV has a singlet. Based on these results and dideoxy sequencing, the *DrdI* islands in these clones are found to have 5 different sequences with AA overhangs (i.e. sequences 1a to 1e) at defined positions in 1 or more of the 4 BAC clones, as shown in Figure 38. Based on this data alone, concordances (i.e. an indication that 2 or more clones are contiguous) are found between clones I and III (i.e. sequence 1b in the triplet in clone I and the doublet of clone III), clones II and III (i.e. sequence 1e in the doublet in clone II and the doublet of clone III), clones III and IV (i.e. sequence 1e in the doublet in clone III and the singlet of clone IV), and clones II and IV (i.e. sequence 1e in the doublet in clone II and the singlet of clone IV). On the other hand, discordances (i.e. an indication that 2 or more clones are not contiguous) are found between clones I and II (i.e. there is no overlap between the 1a, 1b, and 1c sequences of clone I and the 1b and 1e sequences of clone II) and clones I and IV (i.e. there is no overlap between the 1a, 1b, and 1c sequences of clone I and the 1e sequences of clone IV). Based on the identification of these concordances and discordances, a tentative alignment for some of clones I to IV can be determined, as shown at the bottom of Figure 38.

Figure 39 shows how the *DrdI* island approach is used to determine the sequences of sites with AC overhangs and, based upon this information, to tentatively align the 4 hypothetical BAC clones. Using the analysis described above with respect to Figure 38, but for the AC overhangs, 3 concordances and 2 discordances are identified and the tentative alignment of the 4 hypothetical BAC clones is determined, as shown in Figure 39. As noted above, the results of Figure 38 identified concordance among BACS I through IV based on overlapping sequences. However, as shown with respect to Figure 39, a concordance cannot be deduced between BAC I and III, since there are no overlaps in the identified sequences.

Figure 40 shows how the *DrdI* island approach is used to determine the sequences of sites with AG overhangs and, based upon this information, to tentatively align the 4 hypothetical BAC clones. Using the analysis described above with respect to Figure 38, but for the AG overhangs, 2 concordances and 2 discordances are identified and the tentative alignment of the 4 hypothetical BAC clones is determined,

as shown in Figure 40. Overlap between BAC II & III, or BAC III & IV could not be deduced using the AG overhang site alone.

Figure 41 shows how the *Drd*I island approach is used to determine the sequences of sites with CA overhangs and, based upon this information, to tentatively align the 4 hypothetical BAC clones. Using the analysis described above with respect to Figure 38, but for the CA overhangs, 4 concordances and 2 discordances are identified and the tentative alignment of the 4 hypothetical BAC clones is determined, as shown in Figure 41.

Figure 42 shows how the *Drd*I island approach is used to determine the sequences of sites with GA overhangs and, based upon this information, to tentatively align the 4 hypothetical BAC clones. Using the analysis described above with respect to Figure 38, but for the GA overhangs, 1 concordance and 2 discordances are identified and the tentative alignment of only 2 of the 4 hypothetical BAC clones is determined, as shown in Figure 42.

Figure 43 shows how the *Drd*I island approach is used to determine the sequences of sites with GG overhangs and, based upon this information, to tentatively align the 4 hypothetical BAC clones. Using the analysis described above with respect to Figure 38, but for the GG overhangs, no concordances and 1 discordance are identified and the tentative alignment of the 4 hypothetical BAC clones cannot be determined, as shown in Figure 43. In Figure 43, there is a doublet in clone I based on the presence of sequences 6a and 6b, a singlet based on the presence of sequence 6c, and a multiplet in clone III based on the presence of sequences 6a, 6b, 6c, and 6d. In view of multiplet in clone III, the sequence of the *Drd*I island GG overhangs cannot be determined. However, a set of 4 sequencing primers can be used to extend one base beyond the GG overhang (i.e. the 3' end of the primers contains GGA, GGC, GGG, and GGT) to obtain additional information. However, it is not necessary to do so in this case, because the data for the other overhangs shows that concordance exists between clones I and III and between clones III and IV.

The analyses conducted in conjunction with Figures 38 to 43 can be combined to obtain a listing of the sequences obtained for each of the dinucleotide overhangs, a listing of the concordances, and a listing of the discordances, as shown in Figure 44. Based on this information, the unique and overlapping *Drd*I islands in

the 4 hypothetical BAC clones can be identified and the clones themselves aligned, in accordance with Figure 45. In this hypothetical, as illustrated, the order of the clones is as follows: I, III, IV, and II. This result was determined on a very conservative basis. For example, although sequence 6c is unique to clone IV, the multiplet of GG sequences in clone III precludes an unambiguous assignment for the position of this sequence. Also, the listing does not order the *Drd*I sites which are unique to a given clone. Finally, one can arrange the information to achieve a contig of the map position of the *Drd*I sites which correspond to the individual BAC clones. The *Drd*I sites are grouped into 6 sets allowing a rough determination of the BAC clone alignment. Certain sites remain unmapped, such as 6c – although one may surmise that it probably overlaps with clone III, since clone II lacks a *Drd*I site with a GG overhang. The precise order of *Drd*I sites within a grouping cannot be determined from this data alone, but will be easily obtained from sequence information on smaller cosmid clones, once the BAC contig is completed.

Examples of alignment of human DNA BAC contigs using *Drd*I islands

The simulations in the previous section demonstrate how the *Drd*I alignment is achieved. BAC overlaps in the genome databases were rare. The following are examples from 3 contigs on chromosome 7. Figure 46 shows representational fragments which would be obtained with *Drd*I/*Msp*I/*Taq*I digests. Figure 47 shows representational fragments which would be obtained with *Drd*I/*Mse*I digests. The fragments which allow one to establish overlap have appropriate symbols next to them to show that they are in more than one BAC.

For an example using *Drd*I/*Msp*I/*Taq*I digests, contig 1941 contains 3 BACs. BAC RG253B13 overlaps with RG013N12 based on the *Drd*I/*Msp*I/*Taq*I fragments generated from *Drd*I AG (115 and 353 bp), AC (381 bp), CA (559 bp), GA (3,419 bp; may not amplify) and AA (192 and 597 bp) overhangs. BAC RG013N12 overlaps with RG300CO3 based on the *Drd*I/*Msp*I/*Taq*I fragments generated from *Drd*I AG (1,137 bp), CA (16 bp, may be too small), and AA (2,328 bp).

For example, using *Drd*I/*Mse*I digests, contig T002144 contains 5 BACs. BAC RG022J17 overlaps with RG067E13 based on the *Drd*I/*Mse*I fragments

generated from *Drd*I AG (338bp), GA (17, 77, and 586 bp), AA (273 bp), and GG (55 bp) overhangs. BAC RG067E13 overlaps with RG011J21 based on the *Drd*I/*Mse*I fragments generated from *Drd*I AC (71bp). BAC RG011J21 overlaps with RG022C01 based on the *Drd*I/*Mse*I fragments generated from *Drd*I AG (92bp), AA (48 bp), and GG (42 bp) overhangs. Note that establishing overlap between RG022C01 and RGO43K06 would require either using the other *Drd*I overhangs (in this case TT) or, alternatively, having more BACs in the library.

900,000 short sequencing reactions will be needed to create an entire BAC contig using the *Drd*I islands approach: completed in 39 days using 10 of the Perkin Elmer 3700 machines.

As described above, the *Drd*I island procedure is amenable to automation and requires just a single extra reaction (simultaneous cleavage/ligation) compared to dideoxy sequencing. Use of 6 additional primers is compatible with microtiter plate format for delivery of reagents (6 at a time). Further, only very short sequences of 80 to 100 bases are more than sufficient to determine concordance or discordance with other entries into the database.

With an average BAC size of 100-150 kb, a total of 20,000 to 30,000 BAC clones would cover the human genome, or 150,000 clones would provide 5-fold coverage. For each clone, one requires 6 sequencing runs for a total of 900,000 sequencing reactions. At a conservative estimate of 1 run per hour of 96 reactions, with 24 loadings/day, this equals 2,304 sequencing reads/PE 3700 machine/day.

Thus, the *Drd*I approach for overlapping all BAC clones providing a 5-fold coverage of the human genome would require only 39 days using 10 of the new PE 3700 DNA sequencing machines.

The complete set of *Drd*I islands provided six sets to determine overlap. The number of islands within a BAC can be increased by using a second representation, such as *Bgl*I. Further, this example used only 4 hypothetical clones with minimal coverage, in the actual human genome sequencing, there will be a 10-fold coverage of the genome. The precise order of *Drd*I sites within a grouping cannot be determined from this data alone, but will be easily obtained from sequence information on smaller 10 kb plasmid clones, once the BAC contig is completed.

Completing the entire genome sequence based on the BAC *DrdI* and *BglI* islands.

The total unique sequence in the hybrid *DrdI*-*BglI* island database will be approximately 125 million to 210 million anchored bases with an additional 145 million bases of relatively anchored sequences from the BAC ends. This will provide 9.2 to 12% of anchored and relatively anchored genomic sequence, or approximately $1/10^{th}$ of the entire genome will be ordered on the human genome. This is sufficient density to allow for a shotgun sequencing of total genomic DNA from the ends of 10 kb clones. The shotgun cloning will require only a 5-fold coverage of the genome since the islands are relatively dense. At an average of 1 kb reads (i.e. 2 sequencing reactions of 500 bp/clone), 3,000,000 clones would provide 1-fold coverage and 15,000,000 clones would provide a 5-fold coverage. Since sequence information will be obtained from both ends, the process will require almost 200 days.

30,000,000/760 sequencing reads/machine/day = 39,473 machines days/200 machines = 197 days

On average, each 10^{th} clone will immediately overlap with one of the ordered island sequences in the above database (9.2 to 12% of genome). Overlap is determined using unique sequences near the ends of a given island. An algorithm is designed to choose 32 unique bases on each side of the island which is not part of a repetitive sequence. This 32 base sequence will be designated a "Velcro island". Thus, for the 250,000 to 350,000 *DrdI* and *BglI* ordered islands in the database, there will be between 500,000 and 700,000 "Velcro islands". As sequence information is generated, it is queried in 32 bit portions to see if it has either perfect 32/32 or almost perfect 31/32 alignment with one of the Velcro sequences. If yes, then the neighboring 20 bases on each side (if available) are also queried to determine if this is a true overlap. When this overlap is achieved, it generates 3 new "Velcro islands" and removes one of them from the database. One of the new Velcro islands is the distal sequence on the 500 bases which overlap with the original *DrdI* island. The other two new Velcro islands are the end portions of the 500 base sequence attached to this particular clone, either approximately 10 kb upstream, or downstream of the

DrdI island, depending on orientation. If any of the new Velcro regions is in a repeat sequence, it is removed from the Velcro database. This reduces formation of false contigs. These two new Velcro islands are immediately queried against all other *DrdI* and *BglI* islands in the BAC contig region. In the example in Figures 42-43, islands 1e, 2c, and 4c all map to the same contig region. This type of analysis is repeated with each new random plasmid sequence, thus initially creating more Velcro islands, and subsequently creating less Velcro islands as the genomic sequence fills in. Each genome equivalent will hit from 80% to 90% of the Velcro islands, expanding each island by an average of 500 bases, + a bridge of another 500 bases or about 400 to 600 million bases. Thus, on a first pass, ordered information should increase from about 9%-12% to about 21%-32% the genome. The remaining clones are rescanned into the new Velcro database, which now has from 2 to 2.5-fold more islands, allowing more connectivity points which now increase to about 800 to 1,200 million bases, or about 47%-72% the genome and with a third and fourth pass, this approach leads to a complete sequence of the entire genome. The genome is substantially filled in by the 5-fold coverage.

Construction of a finished genomic sequence over a 1 megabase region was simulated using a random number generator which provided sequence read start points for 5,000 "random" clones, with the assumption that each start point provided 500 bases of sequence. To each of these, another 500 bases of sequence was included at a random distance of 8 to 12 kb downstream. The randomly generated sites were sorted by position and queried for presence of sequencing gaps. This was based on the conservative requirement for 40 bp overlap between two sequence runs. Thus, sequence start points more than 460 bases apart were scored as gapped. Two types of gaps need to be considered: (i) Gaps in sequence information between the two 500 bases generated from a random clone, which will be filled in as needed, and (ii) Gaps between two unrelated clones which are not bridged. In the 1 megabase region, there were 74 small gaps which were in-between a given clone. Of these, 50 gaps were between 460 and 560 bases, i.e. less than 100 bases from the nearest anchored sequence. Thus, extending the sequencing read from 500 to 600 bases would close these 50 regions. The remaining 24 sites are less than 500 bp away from an anchored

site and can be filled in when the region in question is being closely scrutinized for important genes.

The 1 megabase region also contained 26 gaps in between two unrelated clones which were not bridged. Of these, 21 were between 460 and 560 bases, i.e. less than 100 bases from the nearest anchored sequence. Thus, extending the sequencing read from 500 to 600 bases would close these 21 regions. The remaining 6 sites need to be filled in using primer walking. Five of these sites were within 500 bp, and the remaining site was within 1,000 bp – thus, each of these regions can be closed using sequencing primers from both sides of the anchored sequence. The same primers are used to PCR amplify the region from the genome and then sequence it. On average, 12 sequencing/PCR primers will be required to close 6 gaps per megabase. For the entire human genome at 3,000 megabases: 3,000 x 12 = 36,000 primers and sequencing runs. There are a number of commercial vendors synthesizing primers, many of whom claim capacity of "1,000's of oligo's per day", so at a conservative estimate of 2,000 primers/day @ $20/primer, the synthesis run would require 18 days.

36,000/760 sequencing reads/machine/day = 47 machines days/200 machines = 0.23 days

The grand total is:

Mapped *DrdI* and *BglI* islands with over 200,000 SNPs; 10-fold coverage of BACs w/ends = 31.5 days
Random 10kb plasmid clones; 5-fold coverage of entire genome = 197 days
Closure of gaps using primer walking = 18.5 days

Total: = 247 days

BAC clone derived singlets are used to align plasmid *DrdI* islands to generate a comprehensive *DrdI* SNP database.

The singlet sequences deduced from deconvoluting the BAC clone contig database (see above) will be used to align more complete *DrdI* islands

generated by sequencing in both directions from cosmid or plasmid clones. About 200,000 to 300,000 *DrdI* islands are predicted in the human genome. The *DrdI* islands are a representation of 1/15th to 1/10th of the genome.

As described above, 500,000 plasmid or cosmid clones of average size 30-40 kb will provide 5 to 6-fold coverage of the human genome. These plasmids and cosmids will be generated from a mixture of 10 individual's DNA to provide a rich source of SNPs. Initially, only 6 primers will be used per plasmid/cosmid to identify those *DrdI* sites present in the clone. A subsequent run will be performed with the correct overhang linkers for generating the sequence of the opposite strand for those *DrdI* sites present in that clone, as well as using more selective primers for obtaining unique sequence information from doublet or triplet reads. An average of 3 sites per clone will rapidly generate 1,500,000,000 bases of sequence information from the *DrdI* sites, plus the 500,000,000 bases of unique sequence information from the ends of the clones. The 1,500,000,000 bases of sequence information from the *DrdI* sites will contain the same regions resequenced an average of 5-6 times providing 250,000,000 to 300,000,000 bases of unique sequence and ample amounts of SNP information. This comprehensive *DrdI* island approach will require on average 12 sequencing runs per clone to determine the unique singlet *DrdI* sequences, for a total of 6,000,000 sequencing runs.

This comprehensive *DrdI* island approach will provide from 250,000 to 430,000 SNPs. It has been estimated that 30,000 to 300,000 SNPs will be needed to map the positions of genes which influence the major multivariate diseases in defined populations using association methods. Further, the above SNP database will be connected to a closed BAC clone map of the entire genome. A more rapid approach to finding SNPs is provided below.

<u>A novel shotgun approach to generate a mapped *DrdI* SNP database, which is amenable to high-throughput detection on a DNA array.</u>

In the above-described procedure for PCR-amplifying the *DrdI* island directly from a BAC clone by using a second frequent cutter enzyme to create small fragments for amplification was described. The second enzyme (e.g. *MspI*) can contain a two base 5' overhang such that ligation/cutting could proceed in a single

reaction tube. The ligation primers/ PCR primers can be designed such that *only* *Drd*I-second enzyme fragments amplify.

A detailed evaluation of 4 sequenced BAC clones from 7q31 shows that ideally, the second enzyme should be a mixture of both *Taq*I and *Msp*I.

*Taq*I is known to retain some activity at 37°C, and, thus, the entire reaction containing DNA, adapter linkers, *Drd*I, *Taq*I, *Msp*I, and T4 ligase may be carried out in a homogeneous reaction at 37°C. Further, *Taq*I becomes irreversibly denatured at 75°C. Therefore, a heat step prior to the PCR reaction is sufficient to inactivate all the enzymes.

A close analysis of the length of fragments generated in a *Drd*I, *Taq*I, and *Msp*I cleavage/ligation/amplification reveals that not every *Drd*I site is amplified (on the assumption that fragments above 4 kb will not amplify well in a mixture containing much smaller amplicons.) Further, in a competition, where one fragment is small (i.e. 200 bp) compared to a much larger fragment (i.e. 2,000 bp), the smaller one will generate more PCR product, which may be sufficient to swamp out the sequencing ladder in the first 200 bases. Ironically, this only aids in the analysis of the sequence information, because comparisons of singlet with singlet reads is the easiest to interpret.

In one BAC clone, RG364P16, the *Drd*I sites are positioned such that the AA, AC, AG, CA, GA, and GG overhangs used in the linker would generate only 3 fragments below about 4,000 bp. Actually, the first site would generate an additional product to a *Taq*I or *Msp*I site within the BAC vector. See Figure 48. Even three sites are sufficient to determine clone overlap. Nevertheless, if needed, linkers containing the complement TT, GT, CT, TG, TC, and CC overhangs would provide additional sequences at some of the other *Drd*I sites.

For creating the representation required for shotgun cloning, 1 μg of pooled genomic DNA (200 ng each from 5 individuals = 10 chromosome equivalents) = 150,000 copies of the genome = 0.25 attomoles of genomes or 0.5 attomoles of each gene is used. This procedure is shown in Figure 49 and is largely the same as that described with reference to Figure 5, except after PCR amplification, the PCR product is cut with *Xma*I and *Xho*I enzymes. The resulting digested product is separated on a gel. The fragments of 200 to 1000 bp are cloned into the corresponding sites of a

vector. The inserts can be sequenced to build a mapped SNP database. This procedure is described in more detail below.

The pooled DNA is cut with *Drd*I, *Taq*I, and *Msp*I, in the presence of phosphorylated *Drd*I adapters containing a unique 2 base 3' overhang (i.e. AA) as well as a methylated *Xma*III site ($C^{m5}CCGGG$) in the adapter sequence, in the presence of unphosphorylated *Taq*I and *Msp*I adapters containing 2 base 5' CG overhangs as well as a methylated *Xho*I site ($CTCG^{m6}AG$) in the adapter sequence, and in the presence of T4 ligase, such that the linkers are added to their respective overhangs in a homogeneous reaction at 37°C. The adapters are methylated so they are not cut by *Taq*I and *Msp*I during this reaction. Enzymes are inactivated by heating at 85°C to 98°C, preferably 95°C, for 2 to 20 minutes, preferably for 5 minutes.

Alternatively, the *Msp*I/*Taq*I adapter is phosphorylated, contains a 3' blocking group on the 3' end of the top strand, and contains a bubble to prevent amplification of unwanted *Msp*I-*Msp*I, *Taq*I-*Msp*I, or *Taq*I-*Taq*I fragments. While the linker can ligate to itself in the phosphorylated state, these linker dimers will not amplify. Phosphorylation of the linker and use of a blocking group eliminates the potential artifactual amplification of unwanted *Msp*I-*Msp*I, *Taq*I-*Msp*I, or *Taq*I-*Taq*I fragments. T4 ligase attaches the *Drd*I and *Msp*I/*Taq*I linkers to their respective sites on the human genome fragments with biochemical selection assuring that most sites contain linkers (See Figure 49A). The adapters are methylated so they are not cut by *Taq*I and *Msp*I during this reaction.

Unmethylated PCR primers are now added in excess of the adapters and used for PCR amplification of the appropriate fragments. Of the approximately 50,000 *Drd*I sites, approximately 70% will give fragments under 4 kb (based on the computer simulation of *Drd*I sites on 4 BAC clones, where 27/38 non-palindromic *Drd*I sites had *Taq*I or *Msp*I sites within 4 kb). Thus, about 35,500 fragments will be amplified. Again, from the simulations, where fragments totaling 24.8 kb are amplified from 550 kb of BAC clone DNA which is 4.5% of the genome, given that only $1/6^{th}$ of those fragments are amplified in a unique overhang representation which is 0.75% representation of the genome. However, for size-selected fragments of between 200 and 1,000 bp, only 15/38 fragments, representing a total of 8.7 kb are

amplified from 550 kb of BAC DNA, and 1/6th of this which is 0.26% representation of the genome (average size of 580 bp; number of fragments is 19,700).

A limited PCR amplification of 11-12 cycles (assuming 90% efficiency per cycle) will give a good representation and produce about 2 μg of final mixed fragments product in the 200- 1,000 bp range, without a major distortion or bias of the representation. The mixed fragments are separated on an agarose gel (i.e. low melting agarose from Seakem) the correct size fragment region excised, purified by standard means, and then cleaved with *Xma*III (heteroschizomer of *Sma*I) and *Xho*I and inserted into the corresponding sites in a standard vector, such as pUC18. The library will contain multiple copies of the approximately 19,700 fragments in the representation. The above procedure can be modified such that the library will contain more or less fragments in the representation. For example, a size-selection between 200 and 2,000 bp will slightly increase the library to approximately 25,000 fragments in the representation. For making larger libraries, more than one linker for the *Drd*I site overhang may be used, e.g. both AA and AC overhangs would double the library to approximately 40,000 fragments in the representation. All the non-palindromic overhangs which are non-complementary (i.e. AA, AC, AG, CA, GA, GG) may be used to make an even larger library of approximately 120,000 fragments in the representation. For making smaller libraries, a PCR primer with one or two additional selective bases on the 3' end is used during the PCR amplification step. For example, use of a *Drd*I site linker with an AA overhang and a PCR primer with an AAC 3' end overhang would reduce the library to approximately 5,000 fragments in the representation. The ideal size of the library will depend on the sequencing capacity of the facility (See Table 7). Other restriction endonucleases with degenerate overhangs as the primary enzyme may be used to create the representational library, such as *Bgl*I, *Dra*III, *Alw*NI, *Pfl*MI, *Acc*I, *Bsi*HKAI, *San*DI, *Sex*AI, *Ppu*I, *Ava*II, *Eco*O109, *Bsu*36I, *Bsr*DI, *Bsg*I, *Bpm*I, *Sap*I, or an isoschizomer of one of the aforementioned enzymes. Palindromic restriction endonucleases may also be used to create the representational library, such as *Bam*HI, *Avr*II, *Nhe*I, *Spe*I, *Xba*I, *Kpn*I, *Sph*I, *Aat*II, *Age*I, *Xma*I, *Ngo*MI, *Bsp*EI, *Mlu*I, *Sac*II, *Bsi*WI, *Pst*I, *Apa*LI, or an isoschizomer of one of the aforementioned enzymes.

Table 7. Shotgun cloning of *DrdI* representation.

DrdI Type	Frequency in Genome	Fragment size (kbp)	# Amplified Sequences	# SNPs in Sequences	Fraction of Genome
AAC	12,500	0.2-1	5,000	4,100	0.07 %
AAC, AAA	25,000	0.2-1	9,850	8,200	0.13 %
AA	50,000	0.2-1	19,700	16,400	0.26 %
AA, AC	100,000	0.2-1	39,400	32,800	0.52 %
6 overhangs	300,000	0.2-1	118,200	98,400	1.56 %

When using shotgun cloning to amplify genomic *DrdI* representations for SNP discovery, it is critical that the amplification procedure does not introduce false SNPs from polymerase errors during amplification. The use of proofreading polymerases such as *Pfu* polymerase should minimize such errors. When creating representational libraries with primer selectivity using a proofreading polymerase, use of probes with 3' thiophosphate linkages is preferred to avoid removal of selective bases from the primer.

An alternative approach to minimize false SNPs is to pre-select the representational fragments, and/or avoid amplification altogether. This may be achieved by using biotinylated linker/adapters to a specific *DrdI* overhang, followed by purification of only those fragments using streptavidin beads. Such primer sequences are listed in Table 8.

Table 8. *DrdI* and *Msp/Taq* Bubble linkers and PCR primers for representational shotgun cloning.

Primer	Sequence (5'→3')
DAA1	5' Biotin-C18 spacer- GAA TAC CCG GGA TGA CTA CGT GTA A 3' (SEQ. ID. No. 40)
DAA2R	5' pA CAC GTA GTC ATC CCG GGT ATT C 3' (SEQ. ID. No. 41)
DAAP3	5' GAA TAC CCG GGA TGA CTA CGT GTsA sA 3' (SEQ. ID. No. 42)
DAC5	5' Biotin-C18 spacer- GAT ACC CGG GAT GAG TAC GAC A 3' (SEQ. ID. No. 43)
DAC6R	5' pT GTC GTA CTC ATC CCG GGT ATC 3' (SEQ. ID. No. 44)
DACP7	5' GAT ACC CGG GAT GAG TAC GAC AsAsC 3' (SEQ. ID. No. 45)
DAG9	5' Biotin-C18 spacer- GAT ACC CGG GAT GAG TAC GTC AAG 3' (SEQ. ID. No. 46)
DAG10R	5' pT GAC GTA CTC ATC CCG GGT ATC 3' (SEQ. ID. No. 47)
DAGP11	5' GAT ACC CGG GAT GAG TAC GTC AsAsG 3' (SEQ. ID. No. 48)
DCA13	5' Biotin-C18 spacer- GAT TAC CCG GGA TGA CTA CGT ATC A 3' (SEQ. ID. No. 49)
DCAGAGG141822R	5' pA TAC GTA GTC ATC CCG GGT AAT C 3' (SEQ. ID. No. 50)
DCAP15	5' GAT TAC CCG GGA TGA CTA CGT ATsCs A 3' (SEQ. ID. No. 51)
DGA17	5' Biotin-C18 spacer- GAT TAC CCG GGA TGA CTA CGT ATG A 3' (SEQ. ID. No. 52)
DGA19	5' GAT TAC CCG GGA TGA CTA CGT ATsG sA 3' (SEQ. ID. No. 53)

DGG21	5' Biotin-C18 spacer- GAT TAC CCG GGA TGA CTA CGT ATG G 3' (SEQ. ID. No. 54)
DGGP23	5' GAT TAC CCG GGT AGA CTA CGT ATsG sG 3' (SEQ. ID. No. 55)
MTCG225	5' GAC ACG TCA CGT CTC GAG TCC TA 3' (SEQ. ID. No. 56)
MTCGp326R	5' pCGT AGG ACT CAC AAC GTG ACG T - Bk (SEQ. ID. No. 57)
MTCGO326R	5' CGT AGG ACT CAC AAC GTG ACG T - Bk (SEQ. ID. No. 58)
MTCG227	5' GAC ACG TCA CGT CTC GAG TCC TsAsC 3' (SEQ. ID. No. 59)
MTCG228	5' GAC ACG TCA CGT CTC GAG TCC TAC 3' (SEQ. ID. No. 60)

Using sufficient starting DNA, the representations may be generated by ligating on biotinylated linkers, removing unreacted linkers, for example, by ultrafiltration on an Amicon YM30 or YM50 filter, and, then, binding only those representational fragments containing the ligated biotinylated linker to streptavidin magnetic beads. After a 30 min. incubation with constant agitation, the captured fragments are purified by magnetic separation, and, then, the complementary strand is melted off the biotinylated strand at 95°C for 30 seconds and rapidly recovered. The single-stranded DNA is converted to double stranded DNA (without methyl groups) using a few (2-5) rounds of PCR with a proofreading polymerase such as Pfu polymerase. Alternatively, non-methylated linkers (listed in Table 9) containing a small mismatch on the biotinylated strand may be used, followed by the above steps of ligation, capture, and purification.

Table 9. New *Drd*I linkers/primers for representational shotgun cloning (no amplification).

Primer		Sequence (5'→3')
DAA101	(New)	5' Biotin-C18 spacer- GAA TAC AAG GGA TGA CTA CGT GTA A 3' (SEQ. ID. No. 61)
DAA102R	(New)	5' pA CAC GTA GTC ATC CCG GGT ATT C 3' (SEQ. ID. No. 62)
DAAP3		5' GAA TAC CCG GGA TGA CTA CGT GTsA sA 3' (SEQ. ID. No. 63)
DAC105	(New)	5' Biotin-C18 spacer- GAT ACA AGG GAT GAG TAC GAC 3' (SEQ. ID. No. 64)
DAC106R	(New)	5' pT GTC GTA CTC ATC CCG GGT ATC 3' (SEQ. ID. No. 65)
DACP7		5' GAT ACC CGG GAT GAG TAC GAC AsAsC 3' (SEQ. ID. No. 66)
DAG109	(New)	5' Biotin-C18 spacer- GAT ACA AGG GAT GAG TAC GTC AAG 3' (SEQ. ID. No. 67)
DAG110R	(New)	5' pT GAC GTA CTC ATC CCG GGT ATC 3' (SEQ. ID. No. 68)
DAGP11		5' GAT ACC CGG GAT GAG TAC GTC AsAsG 3' (SEQ. ID. No. 69)
DCA113	(New)	5' Biotin-C18 spacer- GAT TAC AAG GGA TGA CTA CGT ATC A 3' (SEQ. ID. No. 70)
DCAGAGG141822R2 (New)		5' pA TAC GTA GTC ATC CCG GGT AAT C 3' (SEQ. ID. No. 71)
DCAP15		5' GAT TAC CCG GGA TGA CTA CGT ATsCs A 3' (SEQ. ID. No. 72)
DGA117	(New)	5' Biotin-C18 spacer- GAT TAC AAG GGA TGA CTA CGT ATG A 3' (SEQ. ID. No. 73)
DGA19		5' GAT TAC CCG GGA TGA CTA CGT ATsG sA 3' (SEQ. ID. No. 74)
DGG121	(New)	5' Biotin-C18 spacer- GAT TAC AAG GGA TGA CTA CGT ATG G 3' (SEQ. ID. No. 75)
DGGP23		5' GAT TAC CCG GGT AGA CTA CGT ATsG sG 3' (SEQ. ID. No. 76)

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The resultant single strands are subsequently converted to double strands by extension of a perfectly matched, non-methylated primer using a proofreading polymerase such as *Pfu* polymerase. This procedure avoids PCR amplification altogether, but requires a large amount of starting genomic DNA.

With an average of one SNP every 700 bp, the 19,700 fragments will contain about 16,400 SNPs. To find the most abundant SNPs, a 6-fold coverage of these fragments should suffice. This would amount to 118,400 sequencing runs from one direction and, for clones above 500 bp in length, an additional 50% (59,200 runs) from the other side of the fragment, for a total of 177,600 sequencing runs.

For 500 bp reads, estimating 1 run per 2 hours of 96 reaction, with 12 loadings/day, this equals 1,152 sequencing reads/machine/day. Thus, the shotgun cloning/sequencing of unique *Drd*I islands for finding mapped SNPs in a 6-fold coverage of the human genome would require only 15.4 days using 10 of the new PE 3700 DNA sequencing machines.

For obtaining SNPs using the other 5 representations (AC, AG, CA, GA, and GG), would take an additional 77 days yielding a total of 98,500 SNPs. To double this amount, one would evaluate SNPs using the complement overhangs (TT, GT, CT, TG, TC, and CC), which would require a simultaneous mapping from the original BAC library.

In summary, the entire human genome may be mapped using the *Drd*I island approach, and, using the shotgun representation cloning approach, 197,000 mapped SNPs would be generated in just 88 days using 30 of the PE 3700 DNA sequencing machines.

<u>High-throughput detection of SNPs in a *Drd*I island representation on a DNA array.</u>

A good PCR amplification, starting with 100 pmoles of each primer in 20 µl generates about 3 µg of DNA total about 40 cycles. For a 500 bp fragment, that is about 9 picomoles total = about 0.5 picomoles/µl. However, when PCR amplifying a mixture of fragments, one can generate a larger quantity of product, since product reannealing is the limiting factor in a typical PCR reaction. A good representation can generate 1-2 µg product per µl, or a conservative 20 µg product in a 20 µl

reaction. For a 500 bp fragment, that is about 60 picomoles total = about 3 picomoles/μl. To make a representation for the DNA array, the concept is to selectively amplify a subset of the representation such that sufficient product is formed allowing for LDR discrimination of each SNP allele and addressable array capture/detection.

A procedure for making a representation of genomic DNA which will amplify about 8,750 fragments, of which about 4,100 will contain mapped SNPs for evaluation on a 4,096 address universal addressable array is shown in Figure 49. Start with 100 ng of human DNA = 15,000 copies = 0.025 attomoles of each allele. The DNA is cut with *Drd*I, *Taq*I, and *Msp*I, in the presence of phosphorylated *Drd*I adapters containing a unique two base 3' overhang (i.e. AA) and unphosphorylated *Taq*I and *Msp*I adapters containing two base 5', and in the presence of T4 ligase, such that the linkers are added to their respective overhangs in a homogeneous reaction at 37°C (See Figure 50). Alternatively, the *Msp*I/*Taq*I adapter is phosphorylated, contains a 3' blocking group on the 3' end of the top strand, and contains a bubble. Phosphorylation of the linker and use of a blocking group eliminates the potential artifactual amplification of unwanted *Msp*I-*Msp*I, *Taq*I-*Msp*I, or *Taq*I-*Taq*I fragments. T4 ligase attaches the *Drd*I and *Msp*I/*Taq*I adapters to their respective sites on the human genome fragments with biochemical selection assuring that most sites contain linkers (See Figure 50A). In carrying out this procedure, the initial steps are similar to those shown in Figure 5, up to and including the PCR amplification phase which occurs immediately prior to sequencing, are followed. However, in this procedure, the representation is derived from the total genomic DNA of a biological sample, be it from germline or tumor cells, not from a BAC clone. Further, the PCR primer may have one or two additional base(s) on the 3' end to obtain a representation of the correct # of fragments (about 8,750 in the example provided). In addition, after PCR amplification, the amplification product is subjected to a ligase detection reaction ("LDR") procedure to detect single base changes, insertions, deletions, or translocations in a target nucleotide sequence. The ligation product of the LDR procedure is then captured on an addressable array by hybridization to capture probes fixed to a solid support. This use of LDR in conjunction with the capture of a ligation product on a solid support is more fully described in WO 97/31256 to Cornell

Research Foundation, Inc. and Gerry, N. et al., "Universal DNA Array with Polymerase Chain Reaction/Ligase Detection Reaction (PCR/LDR) for Multiplex Detection of low Abundance Mutations," J. Mol. Biol. 292:251-262 (1999), which are hereby incorporated by reference.

In brief, however, this procedure involves providing a plurality of oligonucleotide probe sets. Each set is characterized by (a) a first oligonucleotide probe, having a target-specific portion and an addressable array-specific portion and (b) a second oligonucleotide probe, having a target-specific portion and a detectable reporter label. The oligonucleotide probes in a particular set are suitable for ligation together when hybridized adjacent to one another on a corresponding target nucleotide sequence, but have a mismatch which interferes with such ligation when hybridized to any other nucleotide sequence present in the sample. The PCR amplification product, described in Figure 50, the plurality of oligonucleotide probe sets, and the ligase are blended to form a mixture which is subjected to one or more ligase detection reaction cycles. The ligase detection reaction cycles include a denaturation treatment, where any hybridized oligonucleotides are separated from the target nucleotide sequences, and a hybridization treatment, where the oligonucleotide probe sets hybridize at adjacent positions in a base-specific manner to their respective target nucleotide sequences, if present in the sample, and ligate to one another to form a ligated product sequence containing (a) the addressable array-specific portion, (b) the target-specific portions connected together, and (c) the detectable reporter label. The oligonucleotide probe sets may hybridize to nucleotide sequences in the PCR amplification product other than their respective target nucleotide sequences but do not ligate together due to a presence of one or more mismatches. As a result, the nucleotide sequences and oligonucleotide probe sets individually separate during the denaturation treatment.

A support with different capture oligonucleotides immobilized at particular sites is used in conjunction with this process. The capture oligonucleotides have nucleotide sequences complementary to the addressable array-specific portions. The mixture, after being subjected to the ligase detection reaction cycles, is contacted with the support under conditions effective to hybridize the addressable array-specific portions to the capture oligonucleotides in a base-specific manner. As a result, the

addressable array-specific portions are captured on the support at the site with the complementary capture oligonucleotide. Reporter labels of the ligated product sequences captured to the support at particular sites are detected. This permits the presence of one or more target nucleotide sequences in the sample to be identified.

The ligase detection reaction process phase of the present invention is preceded by the representational polymerase chain reaction process of the present invention. The preferred thermostable ligase is that derived from *Thermus aquaticus*. This enzyme can be isolated from that organism. M. Takahashi, et al., "Thermophillic DNA Ligase," J. Biol. Chem. 259:10041-47 (1984), which is hereby incorporated by reference. Alternatively, it can be prepared recombinantly. Procedures for such isolation as well as the recombinant production of *Thermus aquaticus* ligase as well as *Thermus themophilus* ligase) are disclosed in WO 90/17239 to Barany, et. al., and F. Barany, et al., "Cloning, Overexpression and Nucleotide Sequence of a Thermostable DNA-Ligase Encoding Gene," Gene 109:1-11 (1991), which are hereby incorporated by reference. These references contain complete sequence information for this ligase as well as the encoding DNA. Other suitable ligases include *E. coli* ligase, T4 ligase, *Pyrococcus* ligase, as well as those listed in Table 3.

The hybridization step, which is preferably a thermal hybridization treatment, discriminates between nucleotide sequences based on a distinguishing nucleotide at the ligation junctions. The difference between the target nucleotide sequences can be, for example, a single nucleic acid base difference, a nucleic acid deletion, a nucleic acid insertion, or rearrangement. Such sequence differences involving more than one base can also be detected. Preferably, the oligonucleotide probe sets have substantially the same length so that they hybridize to target nucleotide sequences at substantially similar hybridization conditions.

The process of the present invention is able to detect nucleotide sequences in the sample in an amount of 100 attomoles to 250 femtomoles. Quantitative detection of G12V mutation of the K-*ras* gene, from 100 attomoles to 30 femtomoles using two LDR probes in the presence of 10 microgram salmon sperm DNA is shown in Figure 51. By coupling the LDR step with a primary polymerase-directed amplification step, the entire process of the present invention is able to detect target nucleotide sequences in a sample containing as few as a single molecule.

Furthermore, PCR amplified products, which often are in the picomole amounts, may easily be diluted within the above range. The ligase detection reaction achieves a rate of formation of mismatched ligated product sequences which is less than .005 of the rate of formation of matched ligated product sequences.

Once the ligation phase of the process is completed, the capture phase is initiated. During the capture phase of the process, the mixture is contacted with the support at a temperature of 45-90°C and for a time period of up to 60 minutes. Hybridizations may be accelerated by adding volume exclusion, chaotropic agents, or Mg^{2+}. When an array consists of dozens to hundreds of addresses, it is important that the correct ligation products have an opportunity to hybridize to the appropriate address. This may be achieved by the thermal motion of oligonucleotides at the high temperatures used, by mechanical movement of the fluid in contact with the array surface, or by moving the oligonucleotides across the array by electric fields. After hybridization, the array may be washed sequentially with a low stringency wash buffer and then a high stringency wash buffer.

It is important to select capture oligonucleotides and addressable nucleotide sequences which will hybridize in a stable fashion. This requires that the oligonucleotide sets and the capture oligonucleotides be configured so that the oligonucleotide sets hybridize to the target nucleotide sequences at a temperature less than that which the capture oligonucleotides hybridize to the addressable array-specific portions. Unless the oligonucleotides are designed in this fashion, false positive signals may result due to capture of adjacent unreacted oligonucleotides from the same oligonucleotide set which are hybridized to the target.

Several approaches have been tested to produce universal addressable arrays. One hundred different 2- and 3-dimensional matrices were tested; the current formulation uses an acrylamide/acrylic acid copolymer containing low levels of bis-acrylamide crosslinker. The polymer surfaces were prepared by polymerizing the monomer solution on glass microscope slides pretreated with a silane containing an acryl moiety. Amino-modified address oligonucleotides containing a hexaethylene oxide spacer were hand-spotted onto NHS pre-activated slides and coupled for 1 hour at 65°C in a humidified chamber. Following coupling, the polymer was soaked in a

high salt buffer for 30 minutes at 65°C to remove all uncoupled oligonucleotides. Both activated and arrayed surfaces can be stored under dry conditions for several months with no decrease in activity.

Hybridization conditions were varied with respect to temperature, time, buffer, pH, organic solvents, metal cofactors, volume exclusion agents, and mixing conditions, using test fluorescently-labeled zip-code complementary probes. Under a variety of conditions, no cross-hybridization was observed between even closely related addresses, with signal-to-noise of at least 50:1. Different addresses hybridize at approximately the same rate yielding approximately the same quantity of fluorescent signal when normalized for oligonucleotide coupled per address. The probes diagrammed in Figure 52 were synthesized and tested in a multiplex PCR/LDR reaction on cell line DNA containing known K-*ras* mutations. Each array identified the mutation correctly with signal-to-noise of at least 20:1 (Figure 53). Further, this demonstrates the ability of the universal array to detect two single-nucleotide polymorphisms simultaneously: the wild-type and mutant sequence are present in all panels except from normal cells or from the cell line containing only the G12V mutant DNA.

The detection phase of the process involves scanning and identifying if ligation of particular oligonucleotide sets occurred and correlating ligation to a presence or absence of the target nucleotide sequence in the test sample. Scanning can be carried out by scanning electron microscopy, confocal microscopy, charge-coupled device, scanning tunneling electron microscopy, infrared microscopy, atomic force microscopy, electrical conductance, and fluorescent or phosphor imaging. Correlating is carried out with a computer.

To determine DNA array capture sensitivity, mixtures of an excess of unlabeled to labeled probe were tested. This simulates an LDR reaction where an excess of unligated probes compete with the labeled LDR products for hybridization to the array. DNA arrays were hybridized in quadruplicate with from 100 amoles to 30 fmol FamCZip13 (synthetic 70-mer LDR product) mixed with a full set of K-*ras* LDR probes (combined total of 9 pmol of discriminating and common probes) under standard conditions. The arrays were analyzed on a Molecular Dynamics FluorImager 595 and an Olympus AX70 epifluorescence microscope equipped with a

Princeton Instruments TE/CCD-512 TKBM1 camera. A signal-to-noise ratio of greater than 3:1 was observed even when starting with a minimum of 3 fmol FamCZip13 labeled-probe within 4,500 fmol Fam label and 4,500 fmol addressable array-specific portion in the hybridization solution (see Figure 54). Using the microscope/CCD instrumentation, a 3:1 signal-to-noise ratio was observed even when starting with 1 fmol labeled product (see Figure 54). Thus, either instruments can readily quantify LDR product formed by either K-*ras* allele at the extremes of allele imbalance (from 6-80 fmol, see Table 11.)

For both instruments, a linear relationship is observed between labeled FamCZip13 added and fluorescent counts captured. Each array was plotted individually, and variation in fluorescent signal between arrays may reflect variation in amount of oligonucleotide coupled due to manual spotting and/or variation in polymer uniformity. Rehybridization of the same probe concentration to the same array is reproducible to +/- 5%, with capture efficiency from 20 to 50%. Since the total of both labeled and unlabeled addressable array-specific portions which complement a given address remains unchanged (at 500 fmol) from LDR reaction to LDR reaction, this result demonstrates the ability to quantify the relative amount of LDR product using addressable array detection. Since the relationship between starting template and LDR product retains linearity over 2 orders of magnitude with a similar limit of sensitivity at about 100 amols (see Figure 51), combining PCR/LDR allele discrimination with array-based detection will provide quantifiable results.

As shown in Figure 50, in embodiment A, the LDR oligonucleotide probe sets have a probe with the discriminating base labeled at its opposite end (i.e. fluorescent groups F1 and F2), while the other probe has the addressable array-specific portion (i.e. Z1). Alternatively, in embodiment B, the LDR oligonucleotide probe sets have a probe with the discriminating base and the addressable array-specific portion at its opposite end (i.e. Z1 and Z2), while the other probe has the label (i.e. fluorescent label F). When contacted with the support, the ligation products of embodiment A are captured at different sites but the same array address and ligation products are distinguished by the different labels F1 and F2. When the support is contacted with the ligation products of embodiment B, the different ligation products all have the same label but are distinguished by being captured at different addresses

on the support. In embodiment A, the ratio of the different labels identifies an allele imbalance. Likewise, such an imbalance in embodiment B is indicated by the fluorescence ratio of label F at the addresses on the support.

In carrying out this procedure, one may start with 100 ng of human DNA = 15,000 copies = 0.025 attomoles of each allele. The DNA is cut with *DrdI*, *TaqI*, and *MspI*, in the presence of phosphorylated *DrdI* adapters containing a unique two base 3' overhang (i.e. AA) and unphosphorylated *TaqI* and *MspI* adapters containing two base 5', and in the presence of T4 ligase, such that the linkers are added to their respective overhangs in a homogeneous reaction at 37°C. Enzymes are inactivated by heating at 85°C to 98°C, preferably 95°C, for 2 to 20 minutes, preferably for 5 minutes. PCR amplification using a primer complementary to the *DrdI* adapter with an additional 3' base, i.e. (3' AAC) and a primer complementary to the other adapter will give a representation of 0.19% of the total genomic DNA.

A PCR amplification of 30 to 35 cycles will give a good representation and produce about 10-20 μg of final mixed fragments. Some variation of thermocycling conditions may be required to obtain a broad representation of the majority of fragments at high yield. The PCR amplification will contain an average of 1.5×10^9 copies for each allele of the approximately 8,750 fragments in the representation. This is equivalent to an average yield of 2.5 fmoles of each product. The larger fragments will yield less PCR product (about 1 fmole each), while the smaller fragments will yield a greater amount of product (from 5–10 fmole each).

The same approach may be used for amplifying SNP containing fragments using either a different base on the 3' end, or alternatively, a different *DrdI* overhang. A total of 24 representation PCR reactions generate the amplicon sets for testing all 98,000 SNPs. Further, fragments amplified in the smaller representation may also be cloned and sequenced to find SNPs.

The above procedure can be modified such that the representation will contain more or less fragments, and/or improve the yield of all fragments. For example, a size-selection between 200 and 2,000 bp prior to PCR amplification may improve the yield of fragments in the representation. For making larger representations, more than one linker for the *DrdI* site overhang may be used, e.g., both AA and AC overhangs, and PCR primers complementary to the *DrdI* adapter

with an additional 3' base (i.e. 3' AAC and 3' ACC) would double the representation to approximately 17,500 fragments. Alternatively, more than one PCR primer complementary to the *Drd*I adapter with an additional 3' base (i.e. 3' AAC and 3' AAT) would also double the representation to approximately 17,500 fragments. Larger representations may be used if PCR amplification generates sufficient product for detection on the above described universal array, and/or as detection sensitivity improves. For making smaller representations, one or two PCR primers with two additional selective bases on the 3' end is used during the PCR amplification step, i.e (3'AAAC + 3'AAAG) would reduce the representation to approximately 4,400 fragments, while use of just one primer (3'AAAC) would reduce the representation to approximately 2,200 fragments. The ideal size of the representation will depend on the number of SNPs which will be detected (See Table 10). Other restriction endonucleases with degenerate overhangs as the primary enzyme may be used to create the representation, such as *Bgl*I, *Dra*III, *Alw*NI, *Pfl*MI, *Acc*I, *Bsi*HKAI, *San*DI, *Sex*AI, *Ppu*I, *Ava*II, *Eco*O109, *Bsu*36I, *Bsr*DI, *Bsg*I, *Bpm*I, *Sap*I, or an isoschizomer of one of the aforementioned enzymes. Palindromic restriction endonucleases may also be used to create the representation, such as *Bam*HI, *Avr*II, *Nhe*I, *Spe*I, *Xba*I, *Kpn*I, *Sph*I, *Aat*II, *Age*I, *Xma*I, *Ngo*MI, *Bsp*EI, *Mlu*I, *Sac*II, *Bsi*WI, *Pst*I, *Apa*LI, or an isoschizomer of one of the aforementioned enzymes.

Table 10: High-throughput detection of SNPs on a DNA array

DrdI Type	Frequency in Genome	# Amplified Sequences	# SNPs in Sequences	Fraction of Genome	Yield of each allele (fmol)
AAAC,	3,125	2,187	1,025	0.05 %	4-40
AAAC, AAAG	6,250	4,375	2,050	0.09 %	2-20
AAC	12,500	8,750	4,100	0.19 %	1-10
AAA, AAC	25,000	17,500	8,200	0.38 %	0.5-5

Large scale detection of SNPs using *Drd*I island representations and DNA array capture.

New technologies to identify and detect SNPs specifically provide tools to further understanding of the development and progression of colon cancer.

One can determine chromosome abnormalities by quantifying allelic imbalance on universal DNA arrays using specific SNPs at multiple loci. This approach has the potential to rapidly identify multiple gene deletions and amplifications associated with tumor progression, as well as lead to the discovery of new oncogenes and tumor suppressor genes.

Competitive and real time PCR approaches require careful optimization to detect 2-fold differences. Unfortunately, stromal contamination may reduce the ratio between tumor and normal chromosome copy number to less than 2-fold. Consider two samples: one with 4-fold amplification of the tumor gene (thick black line) and 50% stromal contamination, the other with loss of heterozygosity (LOH, one chromosome containing the gene is missing, thin black line) and 40% stromal contamination (See Figure 55). Using either microsatellite or SNP analysis, both samples would show an allele imbalance of 2.5 : 1 for the tumor gene (black), and allele balance for the control gene (gray, Figure 55, first line). Comparing the ratio of the tumor gene in the tumor sample to the control gene over the ratio of the tumor gene in the normal sample (normalized to the same number of cells) to the control gene, the stromal contamination reduces the ratio from the amplified sample to 1.75 and increases the ratio from the LOH sample to 0.7 (Figure 55, second line). These ratios are exceedingly difficult to distinguish from 1.0 by competitive PCR. However, by using SNP analysis to compare the ratio of tumor to control allele (i.e. thick line) over the ratio of normal to control allele, then it may be possible to distinguish gene amplification (thick black line) with a ratio of 2.5 from LOH (thin black line) with a ratio of 0.4 (Figure 55, bottom line). It is important that relative allele signal can be accurately quantified.

To determine if PCR/LDR allows accurate quantification of mutant and wild-type K-*ras* alleles, PCR-amplified fragments derived from pure cell lines were mixed in varying ratios and analyzed in a competitive three LDR probe system in which upstream discriminating probes specific for either the wild-type or the G12V mutant allele competed for a downstream probe common to both alleles (Figure 56). Optimal quantification was achieved by using LDR probes in slight excess of K-*ras* template and limiting LDR cycles so products were in the linear range for fluorescent quantification on an ABI 373 sequencer. Under these conditions, mutant/wt ratios

from 1:6 to 6:1 could be accurately quantified, and when normalized to the 1:1 products were within 10% of the predicted value (Table in Figure 56). Similar results were obtained using probe sets for G12D, G12C, and G13D. Quantitative LDR was performed on PCR-amplified DNA isolated from 10 colorectal carcinoma cell lines. Four cell lines contained either pure mutant or wild-type ("wt") alleles, three contained approximately equal amounts of mutant and wt alleles (0.7 - 1.1), and three contained an increased ratio of mutant:wt alleles (1.8-4.0). Allelic imbalance was highly correlated to the proportion of cellular p21 ras protein present in the activated, GTP-bound form. These data support the conclusion that allelic imbalance with amplification of the mutant K-*ras* gene is a second genetic mechanism of K-*ras* activation.

Genomic DNA was extracted from 44 archival primary colon cancers known to contain a point mutation in the K-*ras* gene, amplified using PCR primers specific for exon 1 of K-*ras*, and quantified with competitive LDR. The percentage of stromal cell contamination in primary colon cancers was estimated by an independent pathologist for each sample and this value was used to correct the mutant:wt ratio (Table 11). K-*ras* allelic imbalance was calculated to be 2-fold or greater whenever the corrected mutant/wt ratio measured by LDR exceeded 2 (Table 11). To evaluate the impact of K-*ras* allelic imbalance in this group of patients, disease-specific survival curves were obtained by the Kaplan-Meier method using the log-rank test. While tumors with wild-type or non-amplified K-*ras* mutations (mutant:wt ratio < 2) showed similar survival trends, tumors with amplification of K-*ras* (ratio > 2) had a significantly worse survival compare to mutant tumors without allelic imbalance ($p = 0.03$) and to wt tumors ($p = 0.0001$). Thus, gene amplification is an important second mechanism of K-*ras* activation and negatively impacts on disease-specific survival in colon cancer.

Table 11. Corrected ratios of mutant K-*ras* to wild-type alleles in primary colon cancers.

Representative samples with K-*ras* mutation and low-level allele imbalance (< 2)					Representative samples with K-*ras* mutation and high-level allele imbalance (> 2)				
Tumor #	Genotype	Observed mutant : wt ratio	% Tumor	Corrected mutant : wt ratio	Tumor #	Genotype	Observed mutant : wt ratio	% Tumor	Corrected mutant : wt ratio
11	G12D	0.3	50	0.9	17	G12C	0.6	30	3.4
9	G12C	0.3	40	1.2	27	G12A	0.7	30	4.0
23	G12C	0.4	50	1.2	6	G12V	0.7	30	4.0
12	G12C	0.5	60	1.2	14	G12D	0.9	50	2.7
3	G12V	0.5	50	1.5	29	G12A	1.2	40	4.8
10	G12V	0.5	50	1.5	30	G12D	1.2	50	3.6
37	G12A	0.6	60	1.4	38	G12V	1.3	60	3.0
21	G12D	0.6	50	1.8	13	G12C	1.4	70	2.6
19	G12S	0.6	50	1.8	25	G12V	1.7	30	9.6
31	G12D	0.7	60	1.6	35	G12D	2.0	40	8.0

Colon cancer tumors with known K-*ras* genotype were analyzed to determine the degree of allelic imbalance using a modified PCR/LDR technique. The mutant/wt ratio was determined experimentally and corrected based on the estimated percentage of stromal contamination in the microdissected tumor specimen, using the formula: X = mutant/wt (Observed) x (%T + 2(1-%T)) / %T, where X = Corrected mutant/wt ratio of Chromosomes, and %T = Percentage of tumor cells in section. Allelic imbalance was considered significant when the ratio was more than 2.0 (e.g., at least two copies of the mutant allele compared to one copy of the wt allele in the tumor). For low mutant:wt ratios, allele imbalance may also be due to loss of the normal K-*ras* allele in the tumor cell, e.g., an observed mutant:wt ratio of 0.5 with 50% of the cells from the tumor (samples #3 & #10) may reflect one mutant allele in the tumor cell to two wild-type alleles in the normal cell. Under these calculations X = mutant/wt (Observed) x 2(1-%T) / %T = 0.5 x 2(1-0.5)/0.5 = 1 mutant K-*ras* allele in the tumor cell, with LOH of the other allele. The left side of the table shows representative samples in which allelic imbalance was minimal while the right side of the table shows representative samples in which the K-*ras* mutant allele is amplified. The table demonstrates that the corrected mutant:wt ratio is dependent on both the observed ratio and the percentage of stromal contamination in the sample.

The above data demonstrates that PCR/LDR may be used to accurately quantify mutant and wild-type K-*ras* alleles using an automated DNA sequencer to detect the fluorescent signal. Further, the work above demonstrated that femtomole amounts of CZip fluorescently-labeled product in picomole quantities of total probe and label can be captured at its cognate address and quantified using either FluorImager or CCD detection.

The use of fluorescently-labeled oligonucleotides on DNA arrays have the advantages of multiple labels, long lifetimes, ease of use, and disposal over traditional radiolabels. However, the efficiency of fluorescent emissions from a given

fluorophore is dependent on multiple variables (i.e. solvation, pH, quenching, and packing within the support matrix) which makes it difficult to produce accurate calibration curves. This problem may be effectively circumvented by using two fluorescent labels and determining their ratio for each address (Hacia, et al., "Detection of Heterozygous Mutations in BRCA1 Using High Density Oligonucleotide Arrays and Two-Colour Fluorescence Analysis," Nature Genetics, 14(4):441-7 (1996); DeRisi, et al., "Use of a cDNA Microarray to Analyse Gene Expression Patterns in Human Cancer," Nature Genetics, 14(4):457-60 (1996); Schena, et al., "Parallel Human Genome Analysis: Microarray-Based Expression Monitoring of 1000 Genes", Proc. Nat'l. Acad. Sci. USA, 93(20):10614-9 (1996); Shalon, et al., "A DNA Microarray System for Analyzing Complex DNA Samples Using Two-Color Fluorescent Probe Hybridization," Genome Research, 6(7):639-45 (1996); and Heller, et al., "Discovery and Analysis of Inflammatory Disease-Related Genes Using cDNA Microarrays," Proc. Nat'l. Acad. Sci. USA, 94(6):2150-5 (1997), which are hereby incorporated by reference).

Below two sets of alternative dual labeling strategies are addressed. In the first set, shown in Figure 57, signal is quantified by using a fluorescent label on the array surface at the address. In the second and preferred set, shown in Figure 62, signal is quantified by using a small percentage of fluorescent label on the probe which contains the capture oligonucleotide complement.

The first set of dual label strategies to quantify LDR signal using addressable DNA arrays is shown in Figures 57A-B. In Figure 57A, the common LDR probe for both alleles contains a fluorescent label (F1) and the discriminating probe for each allele contains a unique address-specific portion. Following hybridization of the LDR reaction mixture to an array composed of fluorescently-labeled (F2) ligation product, the ratio of F1/F2 for each address can be used to determine relative percent mutation or allelic imbalance. In Figure 57B, the common probe for both alleles contains an address-specific portion and the discriminating probe for each allele contains a unique fluorescent label, F1 or F2. Following LDR, the reaction mixture is hybridized to an array and the ratios of F1/F2 for each address can again be used to determine relative percent mutation or allelic imbalance. In addition, by adding a third label, F3, to the oligonucleotide coupled to the surface it

will be possible to quantify each allele separately. One method of determining allele imbalance compares ($F1_{captured\ signal}/F2_{address\ signal}$) where the matched tumor and normal samples are hybridized to two different arrays (where variability in addresses is less than 10%, achieved by printing two arrays on the same slide). The allele imbalance is calculated for each sample by the formula $\{(F1_{Allele\ 1:\ tumor}/F2_{Address\ 1}) / (F1_{Allele\ 2:\ tumor}/F2_{Address\ 2})\} / \{(F1_{Allele\ 1:\ normal}/F2_{Address\ 1}) / (F1_{Allele\ 2:\ normal}/F2_{Address\ 2})\}$. Even if considerable variance between addresses remains, the overall calculation for the ratio of allele imbalance will remain accurate, provided the identical reusable array is used for both tumor and normal samples, in which case the above equation simplifies to $(F1_{Allele\ 1:\ tumor}/F1_{Allele\ 1:\ normal}) / (F1_{Allele\ 2:\ tumor}/F1_{Allele\ 2:\ normal})$.

The advantages of using the present invention compared to other detection schemes are as follows: this approach to polymorphism detection has three orthogonal components: (i) primary representational PCR amplification; (ii) solution-phase LDR detection; and (iii) solid-phase hybridization capture. Therefore, background signal from each step can be minimized, and consequently, the overall sensitivity and accuracy of the method of the present invention are significantly enhanced over those provided by other strategies. For example, "sequencing by hybridization" methods require: (i) multiple rounds of PCR or PCR/T7 transcription; (ii) processing of PCR amplified products to fragment them or render them single-stranded; and (iii) lengthy hybridization periods (10 h or more) which limit their throughput. Additionally, since the immobilized probes on these arrays have a wide range of T_ms, it is necessary to perform the hybridizations at temperatures from 0 °C to 44 °C. The result is increased background noise and false signals due to mismatch hybridization and non-specific binding, for example, on small insertions and deletions in repeat sequences. In contrast, the present approach allows multiplexed PCR in a single reaction, does not require an additional step to convert product into single-stranded form, and can readily distinguish all point mutations including polymorphisms in mononucleotide and short dinucleotide repeat sequences. This last property expands the number of polymorphisms which may be considered for SNP analysis to include short length polymorphisms, which tend to have higher

heterozygosities. Alternative DNA arrays suffer from differential hybridization efficiencies due to either sequence variation or to the amount of target present in the sample. By using divergent sequences for the addressable array-specific portion (i.e. zip-code) with similar thermodynamic properties, hybridizations can be carried out at 65°C, resulting in a more stringent and rapid hybridization. The decoupling of the hybridization step from the mutation detection stage offers the prospect of quantification of LDR products, as we have already achieved using gel-based LDR detection.

Arrays spotted on polymer surfaces provide substantial improvements in signal capture compared with arrays spotted directly on glass surfaces. The polymers described above are limited to the immobilization of 8- to 10-mer addresses; however, the architecture of the presently described polymeric surface readily allows 24-mer addresses to penetrate and couple covalently. Moreover, LDR products of length 60 to 75 nucleotide bases are also found to penetrate and subsequently hybridize to the correct address. As additional advantages, the polymer gives little or no background fluorescence and does not exhibit non-specific binding of fluorescently-labeled oligonucleotides. Finally, addresses spotted and covalently coupled at a discrete address do not "bleed over" to neighboring spots, hence obviating the need to physically segregate sites, e.g., by cutting gel pads.

Nevertheless, alternative schemes for detecting SNPs using a primary representational PCR amplification have been considered and are briefly included herein. Since the representations are the consequence of amplification of fragments containing two different adapters, the procedure may be easily modified to render single stranded product which is preferred for "sequencing by hybridization" and single nucleotide polymerase extension ("SNUPE") detection. Thus, one linker adapter may contain a T7 or other RNA polymerase binding site to generate single-stranded fluorescently labeled RNA copies for direct hybridization. Or, one strand may be biotinylated and removed with streptavidin coated magnetic beads. Another alternative option is to put a 5' fluorescent group on one probe, and a phosphate group on the 5' end of the other probe and treat the mixture with Lambda Exonuclease. This enzyme will destroy the strand containing the 5' phosphate, while leaving the fluorescently labeled strand intact.

For detection using single nucleotide polymerase extension ("SNUPE"), a probe containing an addressable array-specific portion on the 5' end, and a target-specific portion on the 3' end just prior to the selective base is hybridized to the target. Fluorescently labeled dye-dioxynucleotides are added with a high fidelity polymerase which inserts the labeled base only if the complementary base is present on the target (Figure 58). The ratios of F1/F2 for each address can be used to determine relative percent mutation or allelic imbalance.

Alternatively, LDR products may be distinguished by hybridizing to gene specific arrays (Figure 59A-B). This may be achieved by hybridizing to the common probe (Figure 59A) or across the ligation junction (Figure 59B). A "universal" nucleotide analog may be incorporated into the address so that neither allele product hybridizes better to the array. Again, the ratios of F1/F2 for each address can be used to determine relative percent mutation or allelic imbalance.

For large representations, or direct detection of any SNPs in the absence of a representation, LDR/PCR may be used (Figure 60). In this scheme, the discriminating probes contain universal probes with unique addressable portions on the 5' side, while the common probes have universal primers on the 3' side. The upstream probe has the addressable array-specific portion in-between the target-specific portion and the universal probe portion, i.e. the probe will need to be about 70 bp long. After an LDR reaction, the LDR products are PCR amplified using the universal PCR primer pair, with one primer fluorescently labeled. To avoid ligation independent PCR amplification, it may be necessary to incorporate a series of blocking groups on the 3' end of the downstream common probe (excellent successes have been achieved by applicants with thiophosphate linkages of the last four O-methyl riboU bases), and treat the ligation products with Exo III. See WO 97/45559, which is hereby incorporated by reference.

The addressable array-specific portion is now in the middle of a double-stranded product. For maximum capture efficiency, it may be desirable to render the product single-stranded, either with T7 RNA polymerase or with biotinylated probe. One alternative option is to put a 5' fluorescent group on one probe, and a phosphate group on the 5' end of the other probe and treat the mix with

Lambda Exonuclease (See Figure 61). This enzyme will destroy the strand containing the 5' phosphate, while leaving the fluorescently labeled strand intact.

The final products are then captured on the addressable array at the specific addresses. The ratio of signal at Z1/Z2 can be used to determine relative percent mutation or allelic imbalance. It may be difficult to quantify subtle differences of allele imbalance since the different addressable array-specific portions may alter the ratio of alleles in the final PCR product. Nevertheless, LDR/PCR may aid in quantification of LOH and gene amplifications at multiple loci simultaneously.

Figure 62 presents the second set of dual label strategies to quantify LDR signal using addressable DNA arrays. In Figure 62A, the common LDR probe for both alleles contains a fluorescent label (F1) and the discriminating probe for each allele contains a unique addressable sequence. A small percentage of each discriminating probe contains a fluorescent label F2. Following hybridization of the LDR reaction mixture to an array, the ratio of F1/F2 for each address can be used to determine relative percent mutation or allelic imbalance. By placing the second fluorescent label on both discriminating probes, one controls for differences in either address spotting or hybridization kinetics of each individual address. For example, consider that 10% of the discriminating probes contain F2. Consider a sample containing 3-fold more of the C allele than the T allele. After an LDR reaction, 20% of the common probe has been ligated to form the T-specific product containing address-specific portion Z1, and 60% has formed the C-specific product containing address-specific portion Z2. Due to differences in spotting, the array captures 50% of the Z1 signal, but only 30% of the Z2 signal. F1/F2 for Z1 = (50% of 20%)/(50% of 10%) = 10%/5% = 2. F1/F2 for Z2 = (30% of 60%)/(30% of 10%) = 18%/3% = 6. By taking the ratio of F1/F2 for Z1 to F1/F2 for Z2, 6/2 = 3 is obtained which accurately reflects the allele imbalance in the sample.

In Figure 62B, the common probe for both alleles contains an addressable sequence and the discriminating probe for each allele contains a unique fluorescent label, F1 or F2. Following LDR, the reaction mixture is hybridized to an array and the ratios of F1/F2 for each address can again be used to determine relative percent mutation or allelic imbalance. In addition, by adding a small percentage of common probe containing label F3, it is possible to quantify each allele separately.

Dual label hybridization to the same address using dye combinations of fluorescein/phycoerythrin, fluorescein/Cy5 Cy3/rhodamine, and Cy3/Cy5 have been used successfully (Hacia, et al., "Detection of Heterozygous Mutations in BRCA1 Using High Density Oligonucleotide Arrays and Two-Colour Fluorescence Analysis," Nature Genetics, 14(4):441-7 (1996); DeRisi, et al., "Use of a cDNA Microarray to Analyse Gene Expression Patterns in Human Cancer," Nature Genetics, 14(4):457-60 (1996); Schena, et al., "Parallel Human Genome Analysis: Microarray-Based Expression Monitoring of 1000 Genes," Proc. Nat'l. Acad. Sci. USA, 93(20):10614-9 (1996); Shalon, et al., "A DNA Microarray System for Analyzing Complex DNA Samples Using Two-Color Fluorescent Probe Hybridization," Genome Research, 6(7):639-45 (1996); and Heller, et al., "Discovery and Analysis of Inflammatory Disease-Related Genes Using cDNA Microarrays," Proc. Nat'l. Acad. Sci. USA, 94(6):2150-5 (1997), which are hereby incorporated by reference). A list of potential dyes which may be used in the labeling schemes described above is provided in Table 12. For the above schemes to be successful, the dye sets used should not interfere with each other.

Table 12: List of Dyes which may be used for fluorescent detection of SNPs.

Dye	Abs. Max (nm)	Em. Max (nm)
Marina Blue	365	460
Flourescein	495	520
TET	521	536
TAMRA	565	580
Rhodamine	575	590
ROX	585	610
Texas Red	600	615
Cy2	489	506
Cy3	550	570
Cy3.5	581	596
Cy5	649	670
Cy5.5	675	694
Cy7	743	767
Spectrum Aqua	433	480
Spectrum Green	509	538
Spectrum Orange	559	588
BODIPY FL	505	515
BODIPY R6G	530	550
BODIPY TMR	545	575
BODIPY 564/6570	565	575
BODIPY 581/591	580	600
BODIPY TR	595	625
BODIPY 630/650	640	650

A representational PCR amplification will contain an average of 1.5 x 10^9 copies of each allele of approximately 8,750 fragments in the representation. This is equivalent to an average yield of 2.5 fmoles of each product. The larger fragments will yield less PCR product (about 1 fmole each), while the smaller fragments will yield a greater amount of product (from 5–10 fmole each). Of these 8,750 fragments, about 4,100 will contain SNPs. As demonstrated above, the representational PCR/LDR/universal array capture scheme should have the requisite sensitivity to detect gene amplification or loss of heterozygosity at the vast majority of these SNPs simultaneously.

This scheme has immediate utility for detecting allele imbalance in tumors. An initial array of 4,096 addresses may be used to find general regions of gene amplifications or LOH. Subsequently, arrays may be used to pinpoint the regions using more closely-spaced SNPs.

A major advantage of the representational PCR amplification is the ability to amplify approximately 8,750 fragments proportionally to their original copy number in the original sample. While some fragments may amplify more than others, repeated amplification of normal samples will reveal fragments whose PCR and LDR products are consistently amplified to similar yields. Thus, for a given fragment which is either amplified or lost in the tumor (designated "g") there will be at least one fragment which retains normal yields (designated "c") For each allele pair (g1, g2) which is imbalanced, there is a control locus (c1, c2) which exhibits heterozygosity in both the normal and tumor sample. To determine if a given allele has been amplified or deleted, the ratio of ratios between matched tumor and normal samples is calculated, e.g., $r = (g1_{tumor}/c1_{tumor}) / (g1_{normal}/c1_{normal})$. If $r > 2$ then g1 is amplified, if $r < 0.5$, then g1 is deleted. The identical calculation is also applied to the matched alleles, g2 and c2 which should yield a value of approximately 1.0, except for cases such as K-*ras*, where one allele may be lost while the other (mutated) allele is amplified. These calculations may be performed with additional informative SNPs in a given region matched with different control regions. Certain SNP/control pairs will amplify at similar rates and, hence, more accurately reflect relative gene copy number.

Examples of the different schemes for distinguishing gene amplification from loss of heterozygosity are illustrated in Figures 63-66. These four figures demonstrate how representational PCR/LDR with addressable array capture may be used to distinguish amplification of genes at the DNA level (Figures 63-64) or, alternatively, loss of one chromosomal region at that gene (LOH, Figures 65-66). Detection of differences using the address complements on the discriminating probes are illustrated in Figures 63 and 65, while placing the address complements on the common probes are illustrated in Figures 64 and 66.

Figures 63-64 illustrate schematically (using pictures of 4 cells) a cancer where the tumor cells (jagged edges) have 4 copies each of one tumor gene allele (C), one copy each of the other tumor gene allele (T), and one copy each of the normal gene alleles (G, A). The normal cells (ovals) have one copy each of the tumor gene alleles (C, T), and one copy each of the normal gene alleles (G, A). By using

representational PCR/LDR with addressable array capture (as described above), one can demonstrate that the one tumor gene allele (C) is present at a higher ratio (i.e. 2.5) than the other tumor gene allele as well as the other normal alleles, even in the presence of 50% stromal contamination. Thus, that allele is amplified.

In particular, after the sample of cells is treated to recover its constituent DNA, which is PCR amplified, the amplified DNA is subjected to an LDR procedure. In Figure 63, the discriminating base is on the oligonucleotide probe with a different addressable array-specific portion for each different discriminating base, while the other oligonucleotide probe is always the same and has the same label. Figure 64 has the discriminating base on the oligonucleotide probe with the label with different labels being used for each different discriminating base, while the other oligonucleotide probe is always the same and has the same addressable array-specific portion. In either case, whether distinguished by hybridization at different array locations using the same label or by hybridization at any location with each ligation product being distinguished and identified by its label, it is apparent that there is a ratio of C to T alleles of 2.5 and a ratio of G to A alleles of 1.0.

Figures 65-66 illustrate schematically (using pictures of 5 cells) a cancer where the tumor cells (jagged edges) have no copies each of one tumor gene allele (T), one copy each of the other tumor gene allele (C), and one copy each of the normal gene alleles (G, A). The normal cells (ovals) have one copy each of the tumor gene alleles (C, T), and one copy each of the normal gene alleles (G, A). By using representational PCR/LDR with addressable array capture (as described above), one can demonstrate that the one tumor gene allele (T) is present at a lower ratio (i.e. 0.4) than the other tumor gene allele as well as the other normal alleles, even in the presence of 40% stromal contamination. Thus, that allele has been lost, i.e. the cell has undergone loss of heterozygosity.

In particular, after the sample of cells is treated to recover its constituent DNA, which is PCR amplified, the amplified DNA is subjected to an LDR procedure. In Figure 65, the discriminating base is on the oligonucleotide probe with a different addressable array-specific portion for each different discriminating base, while the other oligonucleotide probe is always the same and has the same label. Figure 66 has the discriminating base on the oligonucleotide probe with the label with

different labels being used for each different discriminating base, while the other oligonucleotide probe is always the same and has the same addressable array-specific portion. In either case, whether distinguished by hybridization at different array locations using the same label or by hybridization at any location with each ligation product being distinguished and identified by its label, it is apparent that there is a ratio of C to T alleles of 2.5 and a ratio of G to A alleles of 1.0.

For each example, 10% of the probes containing an addressable array-specific portion are labeled with a fluorescent group (F2 in Figures 63 and 65, F3 in Figures 64 and 66). To illustrate that LDR ligation efficiencies are not always identical among two alleles of a given gene, in each example, the ratio of C:T tumor gene allele ligations in the normal cells will be set at 60%:40%; while the ratio of G:A control gene allele ligations in the normal cells will be set at 45%:55%. To simplify the calculations, the chromosomes observed in the illustration will be multiplied by 1,000 to obtain a representative value for the amount of ligation product formed in arbitrary fluorescent units. In addition, the total number of probes containing an addressable array-specific portion in a reaction will be arbitrarily set at 100,000, such that 10% of 100,000 = 10,000 labeled addressable array-specific portion (although not all addresses) will be equally captured. The calculations for the analyses of Figures 63-66 are set forth in Figures 67-70, respectively.

Further, to illustrate that the technique is independent of either array address spotting or hybridization kinetics, the percent of probes captured will be randomly varied between 30% and 60%. This concept will work even in the absence of a "control" fluorescent label on either the addressable array-specific portion (described herein, Figure 62) or fluorescent label on the array addresses. This may be achieved by printing two sets of identical arrays on the same polymer surface side-by-side, where both polymer and amount spotted at each address is relatively consistent, using the first array for the tumor sample, and the second array for the normal control. Alternatively, the same array may be used twice, where results are quantified first with the tumor sample, then the array is stripped, and re-hybridized with the normal sample.

Large scale detection of SNPs using *Drd*I island representations and DNA array capture: Use in association studies.

The above sections emphasized the use of SNPs to detect allelic imbalance and potentially LOH and gene amplification associated with the development of colorectal cancer. The PCR/LDR addressable array scheme may also aid in finding low risk genes for common diseases using "identity by descent" (Lander, E.S., "The New Genomics: Global Views of Biology," Science, 274(5287):536-9 (1996) and Risch, et al., "The Future of Genetic Studies of Complex Human Diseases," Science, 273(5281):1516-7 (1996), which are hereby incorporated by reference). In ethnic populations, chromosomal regions in common among individuals with the same disease may be localized to approximately 2 MB regions using a combination of genome mismatch scanning and chromosomal segment specific arrays (Cheung, et al., "Genomic Mismatch Scanning Identifies Human Genomic DNA Shared Identical by Descent," Genomics, 47(1):1-6 (1998); Cheung, et al., "Linkage-Disequilibrium Mapping Without Genotyping," Nat Genet, 18(3):225-230 (1998); McAllister, et al., "Enrichment for Loci Identical-by-Descent Between Pairs of Mouse or Human Genomes by Genomic Mismatch Scanning," Genomics, 47(1):7-11 (1998); and Nelson, et al., "Genomic Mismatch Scanning: A New Approach to Genetic Linkage Mapping," Nat Genet, 4(1):11-8 (1993), which are hereby incorporated by reference). SNPs near the disease gene (i.e. in linkage disequilibrium) will demonstrate allele imbalance compared with the unaffected population. If the SNP is directly responsible for increased risk, then the allele imbalance will be much higher, e.g., the APCI1307K polymorphism is found in 6% in the general Ashkenazi Jewish population, but at approximately 30% among Ashkenazi Jews diagnosed with colon cancer, who have a family history of colon cancer (Laken, et al., "Familial Colorectal Cancer in Ashkenazim Due to a Hypermutable Tract in APC," Nature Genetics, 17(1):79-83 (1997), which is hereby incorporated by reference). If the actual T -> A transversion responsible for the condition has been identified, then a SNP analysis to demonstrate allele imbalance will be observed by comparing allele frequency in up to 20 unaffected individuals (94% T, 6% A alleles) to those affected individuals with a family history (70% T, 30% A allele).

Alternatively, suppose the SNP is an ancestral G,A polymorphism found on a *DrdI* island near the APC gene (with allele frequencies of 0.5) which predates the founder T → A transversion. Suppose this event occured in the A allele, termed A*, and is in linkage disequilibrium, i.e. recombination has not altered the ancestral haplotype (Lander, E. S., "The New Genomics: Global Views of Biology," Science, 274(5287):536-9 (1996) and Risch et al., "The Future of Genetic Studies of Complex Human Disease," Science, 273(5281):1516-7 (1996), which are hereby incorporated by reference). Then, the allele frequencies are: G = .5 , A = .44, and A* = 0.06. Expanding the formula $(p + q + r)^2 = 1$ gives expected genotype frequencies of GA = 0.44, GG = 0.25, AA = 0.19, GA* = 0.06, AA* = 0.05, and A*A* = 0.004.

To illustrate the predicted allele imbalance at this ancestral G,A polymorphism, compare predicted allele frequencies in 1,000 normal individuals and 1,000 disease individual with a family history of colon cancer. Then for the normals, 1,000 chromosomes will be scored as the G allele and 1,000 chromosomes will be scored as the A allele (containing 880 "A" and 120 "A*"). Among the affected individuals with a family history, approximately 30% (Laken, et al., "Familial Colorectal Cancer in Ashkenazim Due to a Hypermutable Tract in APC," Nature Genetics, 17(1):79-83 (1997), which is hereby incorporated by reference) or 300 individuals contain the A* allele (comprised of GA*, AA*, or A*A*) and the remaining 70% or 700 individuals do not (comprised of GG, AA, or GA). The number of individuals for each genotype is determined by the number of individuals in category x expected genotype frequency / total of genotype frequency in category. For example, the number of individuals with GA = 700 x 0.44 / 0.88 = 350. Other values are: GG = 196; AA = 156; GA* = 159, AA* = 132, and A*A* = 9 (This calculation assumes that A*A* has the same risk as AA*; the number is small enough to be inconsequential). Summation of the number of each allele yields 350 + (196 x 2) + 159 = 901 G alleles and 350 + (156 x 2) + 159 + (132 x 2) + (9 x 2) = 1,099 A alleles, or approximately a 45% G: 55% A allele imbalance. Observation of this imbalance in 400 affected individuals (= 800 alleles) would have a p value of 0.005.

Thus, for isolated populations (e.g., Ashkenazi Jews), evaluation of allele imbalance at ancestral polymorphisms by comparing unaffected with affected

individuals has the potential for identifying nearby genes with common polymorphisms of low risk. Evaluation of multiple SNPs using PCR/LDR with DNA array detection should aid this analysis. Since the SNP arrays are quantitative, it may be possible to determine allele frequency from pooled DNA samples. Allele number from 4 combined individuals may be calculated by quantifying allele ratios, i.e. ratio of 1:1 = 4:4 for the two alleles; ratio of 1:1.67 = alleles of 3:5; ratio of 1:3 = alleles of 2:6; ratio of 1:7 = alleles of 1:7; and if one allele is absent then the other is present on all 8 chromosomes represented in the pooled sample. Such ratios may be distinguished using array detection, which would reduce the above experimental analysis to evaluation of 100 pooled normal and 100 pooled affected samples.

A complete set of about 100,000 SNPs will place a SNP every 30 kb. This would require 25 arrays of 4,096 addresses. When comparing association for 400 disease individuals with 400 normal controls, this would require 20,000 array scans and provide the data on 80,000,000 SNPs in the population. PCR and LDR reactions take 2 hours each, but may be done in parallel. The current scheme would only require 20,000 PCR reactions, followed by 20,000 LDR reactions, and finally 20,000 DNA array hybridizations (1 hr), and scannings (a few minutes per array). This is far more efficient than the current technology which evaluates one SNP at a time.

The SNP DNA array analysis simultaneously provides predicted association for all the affected genes of any prevalent disease (e.g., Alzheimers, heart disease, cancer, diabetis). It will find both positive and negative modifier genes, it will find genes with low penetrance increase for risk, and will map to within 30 kb of all genes which influence the disease. This approach will allow for pinpointing additional polymorphisms within the disease associated genes, opening the prospect for customized treatments and therapies based on pharmacogenomics.

EXAMPLES

Example 1 - Demonstration of T4 DNA Ligase Fidelity in Ligating Linker/Adapters to only their Complementary 2 base 3' Overhangs Using Synthetic Targets.

Ligation reactions with T4 DNA ligase and a variety of linker/adapters (GG-, AA-, AG-, and GA-) and synthetic targets (Tables 13 and 14) were performed to determine the fidelity of T4 DNA ligase under various experimental conditions.

Table 13. *DrdI* and *Msp/Taq* Bubble linkers and PCR primers for BAC clones

Primer	Sequence (5'→3')
BAA29	5' TAG ACT GCG TAC TCT AA 3' (SEQ. ID. No. 77)
BAA3034R	5' pA GAG TAC GCA GTC TAC GAC TCA GG 3' (SEQ. ID. No. 78)
BAAP31	5' CCT GAG TCG TAG ACT GCG TAC TCT AA 3' (SEQ. ID.
BAAP32-FAM	No. 79)
	5' FAM-CCT GAG TCG TAG ACT GCG TAC TCT AA 3' (SEQ. ID. No. 80)
BAC33	5' TAG ACT GCG TAC TCT AC 3' (SEQ. ID. No. 81)
BACP35	5' CCT GAG TCG TAG ACT GCG TAC TCT AC 3' (SEQ. ID. No. 82)
BACP36-FAM	5' FAM-CCT GAG TCG TAG ACT GCG TAC TCT AC 3' (SEQ. ID. No. 83)
BAG37	5' TAG ACT GCG TAC TCA AG 3' (SEQ. ID. No. 84)
BAG37b	5' Biotin-C18-ACT GAG TCG TAG ACT GCG TAC TCA AG 3' (SEQ. ID. No. 85)
BAG38R	5' pT GAG TAC GCA GTC TAC GAC TCA GT 3' (SEQ. ID. No. 86)
BAGP39	5' ACT GAG TCG TAG ACT GCG TAC TCA AG 3' (SEQ. ID. No. 87)
BAGP40-FAM	5' FAM-ACT GAG TCG TAG ACT GCG TAC TCA AG 3' (SEQ. ID. No. 88)
BCA41	5' TAG ACT GCG TAC TCT CA 3' (SEQ. ID. No. 89)
BAC41b	5' Biotin-C18-ACT GAG TCG TAG ACT GCG TAC TCT CA 3' (SEQ. ID. No. 90)
BCA4246R	5' pA GAG TAC GCA GTC TAC GAC TCA GT 3' (SEQ. ID. No. 91)
BCAP43	5' ACT GAG TCG TAG ACT GCG TAC TCT CA 3' (SEQ. ID. No. 92)
BCAP44-FAM	5' FAM-ACT GAG TCG TAG ACT GCG TAC TCT CA 3' (SEQ. ID. No. 93)
BGA45	5' TAG ACT GCG TAC TCT GA 3' (SEQ. ID. No. 94)
BGAP47	5' ACT GAG TCG TAG ACT GCG TAC TCT GA 3' (SEQ. ID. No. 95)

BGAP48-FAM	5' FAM-ACT GAG TCG TAG ACT GCG TAC TCT GA 3' (SEQ. ID. No. 96)
BGG49	5' TAG ACT GCG TAC TAT GG 3' (SEQ. ID. No. 97)
BGG50R	5' pA TAG TAC GCA GTC TAC GAC TCA GT 3' (SEQ. ID. No. 98)
BGGP51	5' ACT GAG TCG TAG ACT GCG TAC TAT GG 3' (SEQ. ID. No. 99)
BGGP52-FAM	5' FAM-ACT GAG TCG TAG ACT GCG TAC TAT GG 3' (SEQ. ID. No. 100)

Table 14. Targets for ligation experiments in synthetic system.

Primer	Sequence (5'→3')
L53FL	5' pCAT TCA GGA CCT GGA TTG GCG A- Fluoroscein 3' (SEQ. ID. No. 101)
TT54R-FAM	5' Fam-TCG CCA ATC CAG GTC CTG AAT GTT 3' (SEQ. ID. No. 102)
CC55R-FAM	5' Fam-TCG CCA ATC CAG GTC CTG AAT GCC 3' (SEQ. ID. No. 103)
CT56-FAM	5' Fam-attaTCG CCA ATC CAG GTC CTG AAT GCT 3' (SEQ. ID. No. 104)
TC57-FAM	5' Fam-attaattaTCG CCA ATC CAG GTC CTG AAT GTC 3' (SEQ. ID. No. 105)

Synthetic targets were fluorescently labeled with Fam and of different lengths such that correct perfect match from unwanted mismatch ligations could be distinguished when separating products on a sequencing gel. Reactions were performed in a 20 μL volume in a modified T4 DNA ligase buffer (20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM dATP, and 2.5 μg/ml BSA) and contained 5 nM ligation target. Products were separated on a denaturing polyacrylamide sequencing gel and quantified using an ABI 373 automated sequencer and GENESCAN software. The effect of T4 DNA ligase enzyme concentration (100 U or 400 U, New England Biolabs units), KCl concentration (50 mM or 100 mM), linker/adapter concentration (50 or 500 nM linker/adapter), temperature (15°C or 37°C), and time (1 hr or 16 hr) on T4 ligase fidelity and activity was examined.

All of the reactions generated the correct ligation product with no detectable misligation product (Figure 71). The total concentration of linker/adapter and KCl concentration sometimes had an effect on overall activity. From these

assays, the optimal conditions for ligation reactions associated with the *Drd*I representational approach was determined to be 100 U T4 DNA ligase (New England Biolabs units), 500 nM linker/adapter, 50 mM KCl, 20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM dATP, and 2.5 μg/ml BSA in a 20 μL reaction incubated at 37°C for 1 h. This condition is the preferred condition, because it is compatible with the restriction enzymes used to generate *Drd*I representations. Although this condition is optimal for T4 DNA ligase, detectable activity was observed under all of the tested combinations of parameters listed above. For other linker adapter sequences of restriction enzyme overhangs, conditions may be optimized using this assay.

Example 2 - Demonstration of Restriction Digestion and Specific Ligation of Linker/Adapters to their Complementary Overhangs Followed by PCR Amplification of the Correct Fragment.

Specificity and reproducibility of *Drd*I Restriction/Ligation/PCR were tested in two vectors (pBeloBAC11 and pBACe3.6) and a BAC clone. BAC DNA (5-10 ng) was digested with *Drd*I, *Msp*I, and *Taq*I and, simultaneously, ligated with 500 nM of the appropriate linker/adapters in the presence of T4 DNA ligase. Linker/adapters containing 2 base 3' overhangs complementary to the *Drd*I site (BAA29 + BAA3034R for AA overhangs, BAC33 + BAA3034R for AC overhangs, BAG37 + BAG38R for AG overhangs, BCA41 + BCA4246R for CA overhangs, BGA45 + BCA4246R for GA overhangs, and BGG49 + BGG50R for GG overhangs) are listed in Table 13. Linker/adapters containing 2 base 5' overhangs complementary to the CG overhang of *Msp*I or *Taq*I sites (MTCG225 + MTCG0326R or MTCGp326R) are listed in Table 8. The MTCG225/MTCG0326R and MTCG225/MTCGp326R linker adapters contain a bubble to avoid unwanted *Msp*I-*Msp*I, *Taq*I-*Msp*I, or *Taq*I-*Taq*I fragment amplifications. This digestion/ligation reaction was performed in a buffer containing 20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 50 mM KCl, 10 mM dithiothreitol, 1 mM dATP, and 2.5 μg/ml BSA. Reactions were incubated at 37°C for one hour followed by an 80°C incubation for 20 min in order to heat inactivate the enzymes. Since *Taq*I is a thermophilic enzyme, 10-

fold more units were used to counterbalance the 10-fold lower activity at 37°C. This enzyme is fully inactivated by the above heating step.

To remove fragments and linkers with sizes smaller than 100 bps, the digestion/ligation reaction was microcentrifuged with an Amicon YM-50. First, the sample was centrifuged at 8000 rpm for 8 min, then the filter was inverted and the desired products were recovered by centrifuging at 6000 rpm for 3 min. After recovery, the sample volume was brought up to 20 µL with ddH_2O for PCR amplification.

PCR reactions contained the YM-50 purified digestion/ligation reaction (20 µl), 1x PCR buffer (10 mM Tris-HCl (pH 8.3), 50 mM KCl), 4 mM $MgCl_2$, 0.4 mM dNTPs, 1.25 U AmpliTaq Gold, and 0.5 µM PCR primers in a 50 µl reaction. The PCR reactions were initially incubated at 95°C for 10 min (to activate AmpliTaq Gold polymerase) followed by 35 cycles of 94°C, 15 sec; 65°C, 2 min.

Assays performed with pBeloBAC11 or pBACe3.6 resulted in even amplification of 2 fragments for GA- overhangs and 1 fragment each for AA- or CA- overhangs as predicted based on the presence of these overhangs in the plasmids. Similar assays were performed with BAC RG253B13 and also generated the expected results (2 fragments for GA- overhangs and 3 fragments for AA- overhangs respectively, see Figure 46). The larger 3,419 bp GA fragment was not observed, because it was not expected to be amplified. These results demonstrate that the restriction digestion was sufficiently complete and the ligation and PCR reactions were specific for the desired products.

Example 3 - Suppression of Amplification of Vector Derived Sequence while Amplifying the Correct Fragment.

The PCR amplification of *DrdI* fragments derived from the vector sequence were suppressed using PNA or propynyl clamping oligos. A slightly modified protocol was used when PCR amplifying *DrdI* fragments containing AA, CA, or GA overhangs from BACs derived from the pBeloBAC11 or pBACe3.6 vector. The pBeloBAC11 and pBACe3.6 vectors both contain *DrdI* sites complementary to AA-, CA-, and GA- overhangs, and amplification of these vector

fragments needed to be suppressed. Clamping oligos which bind specific *DrdI* fragments (i.e. vector derived) and block annealing of PCR primers, were designed as PNA or propynyl derivatives (Tables 5 and 6).

BAC DNA (5-10 ng) was digested with *DrdI*, *MspI*, and *TaqI* and simultaneously ligated with 500 nM of the appropriate linker/adapters in the presence of T4 DNA ligase in a buffer containing 20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 50 mM KCl, 10 mM dithiothreitol, 1 mM dATP, and 2.5 μg/ml BSA. Reactions were incubated at 37°C for one hour followed by an 80°C incubation for 20 min in order to heat inactivate the enzymes. Fragments and excess linker/adapter less than 100 bp were removed by ultrafiltration on Amicon YM50 filters as described above. PCR reactions contained the YM-50 purified digestion/ligation reaction (20 μl), 1x PCR buffer (10 mM Tris-HCl (pH 8.3), 50 mM KCl), 4 mM $MgCl_2$, 0.4 mM dNTPs, 1.25 U AmpliTaqGold, 1 μM of clamping oligos, and 0.5 μM PCR primers in a 50 μl reaction. The PCR reactions were initially incubated at 95°C for 10 min (to activate AmpliTaq Gold polymerase) followed by 35 cycles of 94°C, 15 sec; 65°C, 2 min. *DrdI* Restriction/Ligation/PCR assays were performed with pBACe3.6 and 1 μM clamping oligos. In one reaction, AA- linker/adapters were ligated to digested vector. This sample was PCR amplified in the presence of a AA- clamping oligo specific for suppressing amplification of AA-*DrdI* fragment associated with only the vector sequence. No vector derived PCR product was observed with both the PNA and propynyl clamping oligos. In a subsequent experiment, CA- and AA- linker/adapters were present simultaneously in the digestion/ligation reaction of pBACe3.6. This reaction was then PCR amplified in the presence of 1 μM AA- clamping oligo (either PNA or propynyl derivative). No AA-product was observed with both the PNA and propynyl clamping oligo, but the amplification of the CA- fragment was unaffected by the presence of the AA- clamp. Similar assays were performed with BAC RG253B13 and also generated the expected number of amplified fragments, depending on which clamps were being used. These results demonstrate the ability of PNA or propynyl clamping oligos to specifically suppress amplification of an undesired fragment, while having no measurable effect on the amplification of desired fragments.

Example 4 - Enrichment of *DrdI* Representational Fragments Using Biotinylated Linker/Adapters and Streptavidin Purification.

Creation of a library of representational fragments is required to rapidly sequence those fragments and discover SNPs. While a PCR amplification reaction may enrich for a particular representation, there also is the possibility of generating false SNPs through polymerase error. An approach to minimizing false SNPs is to pre-select the representational fragments, and/or avoid amplification altogether. This may be achieved by using biotinylated linker/adapters to a specific *DrdI* overhang, followed by purification of only those fragments using streptavidin beads.

While genomic DNA will ultimately be used for this task, BAC DNA was used in this example since proof of the correct selection is easily achieved by demonstrating that the correct fragments amplified. BAC DNA (5-10 ng) was digested with *DrdI*, *MspI*, and *TaqI* and simultaneously ligated with 500 nM of the appropriate linker/adapters in the presence of T4 DNA ligase. Linker/adapters containing 2 base 3' overhangs complementary to the *DrdI* site (BAG37b + BAG38R for AG overhangs and BCA41b + BCA4246R for CA overhangs) are listed in Table 13. Linker/adapters containing 2 base 5' overhangs complementary to the CG overhang of *MspI* or *TaqI* sites (MTCG225 + MTCG0326R or MTCGp326R) are listed in Table 8. The MTCG225/MTCG0326R and MTCG225/MTCGp326R linker adapters contain a bubble to avoid unwanted *MspI*-*MspI*, *TaqI*-*MspI*, or *TaqI*-*TaqI* fragment amplifications. This digestion/ligation reaction was performed in a buffer containing 20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 50 mM KCl, 10 mM dithiothreitol, 1 mM dATP, and 2.5 μg/ml BSA. Reactions were incubated at 37°C for one hour followed by an 80°C incubation for 20 min in order to heat inactivate the enzymes. Fragments and excess linker/adapter less than 100 bp were removed by ultrafiltration on Amicon YM50 filters as described above.

The purification procedure was as follows: (streptavidin magnetic beads and the purification protocol were obtained from Boehringer Mannheim, Indianapolis, Indiana) 10 μl of (10μg/μl) magnetic beads were washed three times

with binding buffer TEN_{100} (10 mM Tris-HCl (pH7.5), 1mM EDTA, 100mM NaCl). The sample (YM-50 purified digestion/ligation reaction) volume was brought up to 100 µl in binding buffer and incubated with washed beads for 30 min (constantly shaking using a neutator or rotating platform). The pellet was washed 2 times with TEN_{1000} (10 mM Tris-HCl (pH7.5), 1mM EDTA, 1000mM NaCl) and then washed once in 1x PCR buffer (10 mM Tris-HCl (pH 8.3), 50 mM KCl), 4 mM $MgCl_2$). The sample was eluted in 30 µl 1x PCR buffer by incubating at 95°C for 5 min, capturing the beads in the magnetic stand for 30 sec at 95°C, followed by immediate removal of the supernatant at the bench. After the streptavidin purification, dNTPs (0.4 mM final concentration), PCR primers (0.5 µM final) and ddH_2O is added to the purified sample to increase the volume to 50 µl. AmpliTaqGold (1.25U) is added, with PCR reactions initially incubated at 95°C for 10 min (to activate AmpliTaq Gold polymerase), followed by 35 cycles of 94°C, 15 sec; 65°C, 2 min.

In assays with pBACe3.6, biotinylated CA- linker/adapters, and non-biotinylated AA linker/adapters, streptavidin purification resulted in only the CA-linker fragment being PCR amplified. Conversely, both CA- and AA- linker fragments were amplified in the control assay without the streptavidin purification step. This result demonstrates that streptavidin purification can be utilized to enrich for specific linker/adapter products prior to the PCR amplification.

Example 5 - Amplification of *DrdI* Representations from the *S. cerevisiae* Genome.

The more complex *S. cerevisiae* genome (16 Mb) was chosen as a more complex model system than individual BACs, but still at $1/200^{th}$ the complexity of the human genome. 100 ng of *S. cerevisiae* genomic DNA was subjected to the same protocol as the BAC DNA as described above. Digestion/ligation reactions were PCR amplified using 7 separate primers with either 2 or 3 base selectivity (AC, CA, GA, AG, GG, CAG, and CAT). A fragment appeared as a band above background in the CA- representation, suggesting the presence of a repetitive element. This band was 2- to 4-fold stronger in the CAG representation, yet absent in the CAT representation. This indicates that PCR primers can also be utilized to alter the size

and complexity of a representation. Inclusion of a size filtration step (Amicon YM-50) before PCR amplification resulted in amplification of a broader representation (based on size) as assayed on an agarose gel.

Example 6 - Amplification of *DrdI* Representations from the Human Genome.

Human DNA has a complexity of 3,500 Mb, and is predicted to contain about 300,000 *DrdI* sites. A *DrdI* representation using three bases of selectivity should amplify about 8,750 fragments, yielding about 0.2% of the genome. A *DrdI* representation using four bases of selectivity should amplify about 2,200 fragments, yielding about 0.05% of the genome. 100 ng of human genomic DNA obtained from Boehringer-Mannheim was digested with 10U *DrdI*, 20U *MspI*, and 100U *TaqI* and simultaneously ligated with 500 nM of the appropriate *DrdI* linker/adapter and 1,000 nM of the *MspI/TaqI* linker/adapter in the presence of T4 DNA ligase. Linker/adapters containing 2 base 3' overhangs complementary to the *DrdI* site (BAG37 + BAG38R for AG overhangs, and BCA41 + BCA4246R for CA overhangs) are listed in Table 13. Linker/adapters containing 2 base 5' overhangs complementary to the CG overhang of *MspI* or *TaqI* sites (MTCG225 + MTCG0326R) are listed in Table 8. This digestion/ligation reaction was performed in a buffer containing 20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 50 mM KCl, 10 mM dithiothreitol, 1 mM dATP, and 2.5 μg/ml BSA. Reactions were incubated at 37°C for one hour followed by an 80°C incubation for 20 min in order to heat inactivate the enzymes. Fragments and excess linker/adapter less than 100 bp were removed by ultrafiltration on Amicon YM50 filters as described above.

PCR reactions contained the YM-50 purified digestion/ligation reaction (20 μl), 1x PCR buffer (10 mM Tris-HCl (pH 8.3), 50 mM KCl), 4 mM $MgCl_2$, 0.4 mM dNTPs, 1.25 U AmpliTaqGold, and 0.5 μM PCR primers in a 100 μl reaction. The PCR primer on the *MspI/TaqI* side was MTCG228 and is listed in Table 8. The PCR primers on the *DrdI* side were complementary to the linker/adapter, and had either 3 or 4 bases of specificity (e.g. primer CATP58 = 3 base CAT specificity, primer CAGP59 = 3 base CAG specificity, primer AGAP60 = 3 base AGA specificity, primer AGAP61 = 3 base AGC specificity, primer AGATP62 = 4

base AGAT specificity, primer AGAGP63 = 4 base AGAG specificity, primer CATGP64 = 4 base CATG specificity, and primer CAGTP65 = 4 base CAGT specificity) and are listed in Table 15.

Table 15. PCR primers for representational PCR /LDR/Arrays.

Primer	Sequence (5'→3')
CATP58	5' CT GAG TCG TAG ACT GCG TAC TCT CAT 3' (SEQ. ID. No. 106)
CAGP59	5' CT GAG TCG TAG ACT GCG TAC TCT CAG 3' (SEQ. ID. No. 107)
AGAP60	5' CT GAG TCG TAG ACT GCG TAC TCA AGA 3' (SEQ. ID. No. 108)
AGCP61	5' CT GAG TCG TAG ACT GCG TAC TCA AGC 3' (SEQ. ID. No. 109)
AGATP62	5' CT GAG TCG TAG ACT GCG TAC TCA AGA T 3' (SEQ. ID. No. 110)
AGAGP63	5' CT GAG TCG TAG ACT GCG TAC TCA AGA G 3' (SEQ. ID. No. 111)
CATGP64	5' CT GAG TCG TAG ACT GCG TAC TCT CAT G 3' (SEQ. ID. No. 112)
CAGTP65	5' CT GAG TCG TAG ACT GCG TAC TCT CAG T 3' (SEQ. ID. No. 113)

The "regular PCR" reactions were initially incubated at 95°C for 10 min (to activate AmpliTaq Gold polymerase) followed by 35 cycles of 94°C, 15 sec; 65°C, 2 min. Another set of PCR condition called "touchdown PCR" was tested in addition to the "regular PCR" as described previously. The "touchdown PCR" protocol consisted of heating for 10 min at 95°C followed by 8 cycles of denaturing for 15 sec at 94°C, annealing/extension for 2 min at 72°C. The annealing/extension temperature was reduced 1°C for each cycle until a final temperature of 64°C. Another 30 cycles of PCR were performed with denaturing 15 sec at 94°C and annealing/extension for 2 min at 64°C. Each sample was performed in quadruplicate, and the 400 µl PCR

products were pooled and concentrated by ultrafiltration on Amicon YM50 filters as described above. Final samples were brought up in 20 µl TE.

PCR amplification of human genome representations (CA- or AG-linker/adapters) were performed with a variety of 3 and 4 base selection primers (e.g., CAG, CAT, CAGT, CATG, AGC, AGA, AGAT, and AGAG). The agarose gel analysis demonstrated apparently equal and broad representation for each of the above PCR primers (Figure 72).

To verify that these human genomic *Drd*I representations were selecting the appropriate fragments, LDR assays were performed to probe for specific fragments within a given representation. LDR conditions used 4 µl of the concentrated representational fragments from the above mentioned PCR reactions, 1x *Tth* DNA ligase buffer (20 mM Tris-HCl pH 8.5, 5 mM $MgCl_2$, 100mM KCl, 1 mM DTT, 1.25 mM NAD^+), 2.5 nM LDR probes. *Tth* DNA ligase (in buffer containing 10mM Tris-HCl pH8.0, 1mM EDTA, 1mg/ml BSA) was added to the reaction to a final concentration of 5 nM. The LDR reaction was carried out with 20 cycles of heating at 95°C for 15 sec and ligation at 64°C for 2 min. Three microliters of the LDR reaction product was loaded on the gel and the gel image was read by GeneScan Analysis 2.02. Control assays containing PCR products generated from primers (Tables 16 and 17) designed for each of the targeted regions demonstrated the integrity of LDR assays (Figure 73).

Table 16. Primers Designed for Detection of Polymorphisms Near *Drd*I Sites by PCR/LDR.

Primer	Sequence (5'→3')
Uni A primer	GGAGCACGCTATCCCGTTAGAC (SEQ. ID. No. 114
Uni B2 primer	CGCTGCCAACTACCGCACATC (SEQ. ID. No. 115
B13 AGA fp1	GGAGCACGCTATCCCGTTAGACCCCTGCAATGACTCCCCATTTC (SEQ. ID. No. 116)
B13 AGA rp1	CGCTGCCAACTACCGCACATCAGTAGGGCTGGGGCATCAGAAC (SEQ. ID. No. 117)
B13 AGA Fam1 (F-1)	Fam aGCTTCAGACACACCAGGCAC =47 (SEQ. ID. No. 118)
B13 AGA -Com1 (C-1)	pATTTAGTTCTTCCTTCTTGCCTCTGC-Bk (SEQ. ID. No. 119)

B13 AGC fp2	**GGAGCACGCTATCCCGTTAGAC**ATTGTGGAAGACAGTGTGGTGAT TC (SEQ. ID. No. 120)
B13 AGC rp2	**CGCTGCCAACTACCGCACATC**CATGGCATATATGTGCCACATTTT C (SEQ. ID. No. 121)
B13 AGC Fam2 (F-2)	FamAAGCATGCTGCTGTAAAGACACA =52C (SEQ. ID. No. 122)
B13 AGC -Com2 (C-2)	PTGCACATGTATGTTTATTGCAGCACTATT-Bk (SEQ. ID. No. 123)
E19 AGC fp3	**GGAGCACGCTATCCCGTTAGAC**GTGTTAGCCAGGATGGTCTCCAT C (SEQ. ID. No. 124)
E19 AGC rp3	**CGCTGCCAACTACCGCACATC**CATGGGTGGGGTAACAGAAAGAAA C (SEQ. ID. No. 125)
E19 AGC Fam3 (F-3)	FamGACAATTATCCTGATTTGGGACC =48C (SEQ. ID. No. 126)
E19 AGC -Com3 (C-3)	pTTACCTTCAGATGGTTTTCCCTCCT-Bk (SEQ. ID. No. 127)
C03 AGA fp4	**GGAGCACGCTATCCCGTTAGAC**TAGTGTCTAGGGATAGAGGAGAA C (SEQ. ID. No. 128)
C03 AGA rp4	**CGCTGCCAACTACCGCACATC**CTCCTGACATTATGGAGAGCCTTA C (SEQ. ID. No. 129)
C03 AGA Fam4 (F-4)	FamAATGCCACACTTCAGATTTTGATAC =50 (SEQ. ID. No. 130)
C03 AGA -Com4 (C-4)	pTTGCAGGATCCTATTTCTGGCACTA-Bk (SEQ. ID. No. 131)

Primer	Sequence (5'→3')
UniAprimer	**GGAGCACGCTATCCCGTTAGAC** (SEQ. ID. No. 132)
UniB2primer	**CGCTGCCAACTACCGCACATC** (SEQ. ID. No. 133)
P20 AGA fp5	**GGAGCACGCTATCCCGTTAGAC**GGACTTCTCCCCACTACAACATA GATTC (SEQ. ID. No. 134)
P20 AGA rp5	**CGCTGCCAACTACCGCACATC**TTTATCAGCAACATGAAAACAGAC TAAC (SEQ. ID. No. 135)
P20 AGA Fam5 (F-5)	FamTGTGGAATTTATCATTTAATTTAGCTTC =56 (SEQ. ID. No. 136)
P20 AGA -Com5 (C-5)	pAGTGAACCGTTCTTTCCAGATTATTTTG-Bk (SEQ. ID. No. 137)
K23 AGA fp6	**GGAGCACGCTATCCCGTTAGAC**AGAATAGAATGCTTGCAATTGAT CAC (SEQ. ID. No. 138)
K23 AGA rp6	**CGCTGCCAACTACCGCACATC**ATGTCAATTTGTTGGGGTTATACA AC (SEQ. ID. No. 139)

K23 AGA Fam6 (F-6)	Fam aaaaAGGAGGGTGACAGTGAACCTG =53 (SEQ. ID. No. 140)
K23 AGA -Com6 (C-6)	pGAGGTAAAATTCAACAATTCATTTGCTT-Bk (SEQ. ID. No. 141)
J17 AGA fp7	GGAGCACGCTATCCCGTTAGACGTGCAGACAAGAGAATGTCAAGT TTC (SEQ. ID. No. 142)
J17 AGA rp7	CGCTGCCAACTACCGCACATCAGAGGCTGGAAAAATAAATCCAAT ACA (SEQ. ID. No. 143)
J17 AGA Fam7 (F-7)	FamGATCAGAAACCACAGGAAATTTG =44 (SEQ. ID. No. 144)
J17 AGA -Com7 (C-7)	pATTTATGCCAGCCCTGCATCCC-Bk (SEQ. ID. No. 145)
AGATP62	CTGAGTCGTAGACTGCGTACTCTAGAT (SEQ. ID. No. 146)
AGAGP63	CTGAGTCGTAGACTGCGTACTCTAGAG (SEQ. ID. No. 147)
CATGP64	CTGAGTCGTAGACTGCGTACTCTCATG (SEQ. ID. No. 148)
CAGTP65	CTGAGTCGTAGACTGCGTACTCTCAGT (SEQ. ID. No. 149)

Table 17. Primers designed for detection of polymorphisms near *DrdI* sites by PCR/LDR/Array Hybridization.

Primer	Sequence (5'→3')
Uni A primer	GGAGCACGCTATCCCGTTAGAC (SEQ. ID. No. 150)
Uni B2 primer	CGCTGCCAACTACCGCACATC (SEQ. ID. No. 151)
GS056H18.2 forward	GGAGCACGCTATCCCGTTAGACGATGAGCTTACACAGGCACTGATTAC (SEQ. ID. No. 152)
GS056H18.2 reverse	CGCTGCCAACTACCGCACATCTATTGGTGACTGATGAAAATGTCAAAC (SEQ. ID. No. 153)
GS056H18.2	Fam-tGTCAAGAAAGTGTATTTAGCTTACAAAC =58 (SEQ. ID. No. 154)
GS056H18.2 -Com2	PTATTAACAGCCTGTTTTACCCTACTTTTG-Bk (SEQ. ID. No. 155)
RG083J23 forward	GGAGCACGCTATCCCGTTAGACGCACCTTATCTTGGCTTTTCTATTC (SEQ. ID. No. 156)
RG083J23 reverse	CGCTGCCAACTACCGCACATCAAGCATATTACATCATGTCATCACTTC (SEQ. ID. No. 157)
RG083J23	Fam-TTCGTTTCTCTTTATCCACACC =52 (SEQ. ID. No. 158)
RG083J23 -Com3	pATGGGAAATGTCTTTTACAATGTACATAAC-Bk (SEQ. ID. No. 159)
RG103H13 forward	GGAGCACGCTATCCCGTTAGACCAGCCATGTGATTCCCTGTGTAC (SEQ. ID. No. 160)

RG103H13 reverse	**CGCTGCCAACTACCGCACATC**CTGCATTGTACAATGCATGCATAC (SEQ. ID. No. 161)
RG103H13	Fam-aaatataaACTAAATGAATCAAAGATAGAGTGAATG =60 (SEQ. ID. No. 162)
RG103H13-Com4	pTATGCATGCATTGTACAATGCAGG-Bk (SEQ. ID. No. 163)
RG103H13.2 forward	**GGAGCACGCTATCCCGTTAGAC**TTCTGATAGAGTCGTTTTGTGCTTC (SEQ. ID. No. 164)
RG103H13.2reverse	**CGCTGCCAACTACCGCACATC**CATTTTAGGATCTGGGAAGCATTAC (SEQ. ID. No. 165)
RG103H13.2	Fam-TTTTTCCTCCCATCCAAATTC =46 (SEQ. ID. No. 166)
RG103H13.2-Com5	pAGAGACCCTAGAATTCTAGCGATGG-Bk (SEQ. ID. No. 167)

Primer	Sequence (5'→3')
UniAprimer	**GGAGCACGCTATCCCGTTAGAC** (SEQ. ID. No. 168)
UniB2primer	**CGCTGCCAACTACCGCACATC** (SEQ. ID. No. 169)
RG118D07 forward	**GGAGCACGCTATCCCGTTAGAC**CCTTGGAAAGCAGGTGCAAATC (SEQ. ID. No. 170)
RG118D07 reverse	**CGCTGCCAACTACCGCACATC**AAATAACAACTGCATTACTCCATCATC (SEQ. ID. No. 171)
RG118D07	Fam-aaTGAAAAAATCCAATATTGGTCTG =55 (SEQ. ID. No. 172)
RG118D07 Com6	pTGTGTGAAAGTGTAAATGTATACGTGTATG-Bk (SEQ. ID. No. 173)
RG343P13 forward	**GGAGCACGCTATCCCGTTAGAC**CTGTCAAGCAGGGAATTGGATAC (SEQ. ID. No. 174)
RG343P13 reverse	**CGCTGCCAACTACCGCACATC**CCTTTCTGATTTCAGTTGCTAGTTTC (SEQ. ID. No. 175)
RG343P13	Fam-GAGACCAAACCAGGGAGAAAG =50 (SEQ. ID. No. 176)
RG343P13-Com-7	pTACAGAGAGAGAGCAAAGAGAGTTCAGAC-Bk (SEQ. ID. No. 177)
RG363E19.2 forward	**GGAGCACGCTATCCCGTTAGAC**TGGAGGTCCTAGCCAGAGCAAC (SEQ. ID. No. 178)
RG363E19.2 reverse	**CGCTGCCAACTACCGCACATC**GGTATTGCCTTTCTGATTTAGCTTTC (SEQ. ID. No. 179)
RG363E19.2	Fam-aGCCCAAAAGCTCCTTCAGC =48 (SEQ. ID. No. 180)
RG363E19.2-Com-9	pTGATAAACAACTTCAGCAAAGTTTCAGG-Bk (SEQ. ID. No. 181)

In addition, these control PCR products were diluted up to 10,000-fold into 10 μg salmon sperm DNA. Even in this vast excess of noncomplementary DNA, LDR assays still identified the desired products.

The targeted *Drd*I- *Msp*I/*Taq*I fragments ranged in size from 130 to 1,500 bp and were derived from AG- or CA- linker/adapters. LDR assays of the human representational libraries demonstrated that the representations were even and that increasing base reach-in generated a more specific library (Figures 74 and 75). This result demonstrates that LDR is sensitive enough to identify a specific *Drd*I-*Msp*I/*Taq*I fragment within a given representation.

Altering the PCR conditions to "touchdown" amplification resulted in more LDR product with no apparent change in the relative distribution of fragments. These results demonstrated that the *Drd*I representational approach was able to generate an even and specific representation of the human genome.

Although the invention has been described in detail for the purpose of illustration, it is understood that such detail is solely for that purpose, and variations can be made therein by those skilled in the art without departing from the spirit and scope of the invention which is defined by the following claims.

WHAT IS CLAIMED:

1. A method of assembling genomic maps of an organism's DNA or portions thereof comprising:

providing a library of an organism's DNA, wherein individual genomic segments or sequences are found on more than one clone in the library;

creating representations of the genome;

generating nucleic acid sequence information from the representations;

analyzing the sequence information to determine clone overlap from a representation; and

combining clone overlap and sequence information from different representations to assemble a genomic map of the organism.

2. A method according to claim 1, wherein said creating representations of the genome comprises:

creating a representation of the genomic segments in individual clones by selecting a subpopulation of genomic segments out of a larger set of the genomic segments in that clone.

3. A method according to claim 2, wherein said selecting a subpopulation of genomic segments comprises:

subjecting an individual clone to a first restriction endonuclease under conditions effective to cleave DNA from the individual clone so that a degenerate overhang is created in the clone and

adding non-palindromic complementary linker adapters to the overhangs in the presence of ligase and the first restriction endonuclease to select or amplify particular fragments from the first restriction endonuclease digested clone as a representation, whereby sufficient linker-genomic fragment products are formed to allow determination of a DNA sequence adjacent the overhang.

4. A method according to claim 3, wherein the first restriction endonuclease creates 2 base degenerate overhangs in the clone and 1 to 12 non-palindromic linker adapters, which contain single stranded overhangs of the formula NN/N'N' where NN/N'N' is selected from the group consisting of AA/TT, AC/GT, AG/CT, CA/TG, GA/TC, and GG/CC, are used.

5. A method according to claim 4, wherein 4 to 6 non-palindromic adapters are used.

6. A method according to claim 3, wherein the first restriction endonuclease creates 3 base degenerate overhangs in the clone and 1 to 16 non-palindromic complementary linker adapters, which contain single stranded overhangs of the formulae NAA, NAC, NAG, NAT, NCA, NCC, NCG, NCT, NGA, NGC, NGG, NGT, NTA, NTC, NTG, and NTT, with N being any nucleotide, are used.

7. A method according to claim 6, wherein 5 to 9 non-palindromic linker adapters are used.

8. A method according to claim 3, wherein the first restriction endonuclease is selected from the group consisting of *Drd*I, *Bgl*I, *Dra*III, *Alw*NI, *Pfl*MI, *Acc*I, *Bsi*HKAI, *San*DI, *Sex*AI, *Ppu*I, *Ava*II, *Eco*O109, *Bsu*36I, *Bsr*DI, *Bsg*I, *Bpm*I, *Sap*I, and isoschizomers thereof.

9. A method according to claim 3, wherein said generating nucleic acid sequence information from the representations comprising:

using sequencing primers to obtain sequence information from the ends of a subpopulation of genomic segments out of a larger set of genomic segments.

10. A method according to claim 9, wherein the sequencing primers have a 5' sequence that is complementary to the adapter primers and have a 3' sequence that is complementary to two or more bases in the degenerate overhang

and/or adjacent to the restriction site recognition sequence to obtain sequencing information adjacent to the restriction site.

11. A method according to claim 10, wherein 1 to 12 sequencing primers are used with a 3' end from the set which end in NN, with N being any nucleotide, and/or it's complement N'N'.

12. A method according to claim 3, wherein 1 to 16 sequencing primers are used with a 3' end from the set which end in NAA, NAC, NAG, NAT, NCA, NCC, NCG, NCT, NGA, NGC, NGG, NGT, NTA, NTC, NTG, and NTT, with N being any nucleotide.

13. A method according to claim 2, wherein said selecting a subpopulation of genomic segments comprises:

subjecting an individual clone to a first restriction endonuclease under conditions effective to cleave DNA from the individual clone so that a palindromic overhang is created in the clone; and

adding complementary linker adapters to the overhangs in the presence of ligase and the first restriction endonuclease to amplify particular fragments from the first restriction endonuclease digested clone as a representation whereby sufficient linker-genomic fragment products are formed to allow determination of a DNA sequence adjacent the overhang.

14. A method according to claim 13, wherein the first restriction endonuclease is *Bam*HI, *Avr*II, *Nhe*I, *Spe*I, *Xba*I, *Kpn*I, *Sph*I, *Aat*II, *Age*I, *Xma*I, *Ngo*MI, *Bsp*EI, *Mlu*I, *Sac*II, *Bsi*WI, *Pst*I, *Apa*LI, or isoschizomers thereof.

15. A method according to claim 13, wherein said generating nucleic acid sequence information from the representations comprising:

using sequencing primers to obtain sequence information from the ends of a subpopulation of genomic segments out of a larger set of genomic segments.

16. A method according to claim 15, wherein the sequencing primers have a 5' sequence that is complementary to the adapter primers and have a 3' sequence that is complementary to two or more bases adjacent to a restriction site recognition sequence to obtain sequencing information adjacent to the restriction site.

17. A method according to claim 2, wherein said selecting a subpopulation of genomic segments comprises:

subjecting an individual clone to a first restriction endonuclease under conditions effective to cleave DNA from the individual clone so that a first non-palindromic overhang is created in the clone;

subjecting an individual clone to one or more second restriction endonuclease under conditions effective to cleave DNA from the individual clone so that a second overhang different from the first overhang is created in the clone;

adding complementary linker adapters to the first and second overhangs in the presence of ligase, the first restriction endonuclease, and the one or more second restriction endonuclease to amplify particular fragments from the restriction endonuclease digest as a representation, whereby sufficient linker-genomic fragment products are formed to allow determination of DNA sequences adjacent to the overhangs.

18. A method according to claim 17, wherein the first restriction endonuclease creates 2 base degenerate overhangs in the clone and 1 to 12 non-palindromic linker adapters, which contain single stranded overhangs of the formula NN/N'N' where NN/N'N' is selected from the group consisting of AA/TT, AC/GT, AG/CT, CA/TG, GA/TC, and GG/CC, are used.

19. A method according to claim 18, wherein 4 to 6 non-palindromic adapters are used.

20. A method according to claim 17, wherein the first restriction endonuclease creates 3 base degenerate overhangs in the clone and 1 to 16 non-

palindromic complementary linker adapters, which contain single stranded overhangs of the formulae NAA, NAC, NAG, NAT, NCA, NCC, NCG, NCT, NGA, NGC, NGG, NGT, NTA, NTC, NTG, and NTT, with N being any nucleotide, are used.

21. A method according to claim 20, wherein 5 to 9 non-palindromic linker adapters are used.

22. A method according to claim 17, wherein the first restriction endonuclease is selected from the group consisting of *Drd*I, *Bgl*I, *Dra*III, *Alw*NI, *Pfl*MI, *San*DI, *Sex*AI, *Ppu*I, *Ava*II, *Eco*O109, *Bsu*36I, *Bsr*DI, *Bsg*I, *Bpm*I, *Sap*I, and an isoschizomer thereof and the one or more second restriction endonuclease is *Mae*II, *Msp*I, *Bfa*I, *Hha*I, *Hin*P1I, *Csp*6I, *Taq*I, *Mse*I, or an isoschizomer thereof.

23. A method according to claim 17, wherein said generating nucleic acid sequence information from the representations comprising:

using sequencing primers to obtain sequence information from the ends of a subpopulation of genomic segments out of a larger set of genomic segments.

24. A method according to claim 23, wherein (1) the sequencing primers have a 5' sequence that is complementary to the adapter primers of the first restriction site and have a 3' sequence that is complementary to two or more bases in the degenerate overhang and/or adjacent to a first restriction site recognition sequence to obtain sequencing information adjacent to the first restriction site and/or (2) the sequencing primers have a 5' sequence that is complementary to the adapter primers of one or more second restriction site and have a 3' sequence that is complementary to two or more bases adjacent to the one or more second restriction site recognition sequence to obtain sequencing information adjacent to the one or more second restriction site.

25. A method according to claim 17, wherein 1 to 12 sequencing primers are used to obtain sequence information adjacent to the first restriction

endonuclease site with the sequencing primers having a 3' end from the set which end in NN, with N being any nucleotide, and/or it's complement N'N'.

26. A method according to claim 17, wherein 1 to 16 sequencing primers are used to obtain sequence information adjacent to the first restriction endonuclease site with the sequencing primers having a 3' end from the set which ends in NAA, NAC, NAG, NAT, NCA, NCC, NCG, NCT, NGA, NGC, NGG, NGT, NTA, NTC, NTG, and NTT, with N being any nucleotide.

27. A method according to claim 1, wherein said generating nucleic acid sequence information from the representations comprising:

using sequencing primers to obtain sequence information from the ends of a subpopulation of genomic segments out of a larger set of genomic segments.

28. A method according to claim 27, wherein 1 to 16 sequencing primers are used to obtain sequence information adjacent to the first restriction endonuclease site with the sequencing primers having a 3' end from the set which ends in NAA, NAC, NAG, NAT, NCA, NCC, NCG, NCT, NGA, NGC, NGG, NGT, NTA, NTC, NTG, and NTT, with N being any nucleotide.

29. A method according to claim 27, wherein unique sequencing data is generated for a unique target known as a singlet sequencing run.

30. A method according to claim 27, wherein two overlapping sequences are generated for two targets known as a doublet sequencing run.

31. A method according to claim 27, wherein three overlapping sequences are generated for three targets known as a triplet sequencing run.

32. A method according to claim 27, wherein the sequencing primer has one or two additional bases on its 3' end to obtain unique singlet sequence information from two or more overlapping sequences.

33. A method according to claim 1, wherein said analyzing sequence information comprises:

analyzing sequencing data generated from representations to deconvolute singlet, doublet and triplet sequencing runs and to determine clone overlap.

34. A method according to claim 33, wherein two singlet sequencing runs in the same representation set from separate genomic clones are compared, said method further comprising:

evaluating the two singlet sequencing runs for clone overlap by aligning the sequencing runs and scoring identity in at least 8 bases beyond the endonuclease recognition site with less than 3 discordant positions.

35. A method according to claim 33, wherein a singlet and a doublet sequencing run in the same representation set from separate genomic clones are compared, said method further comprising:

evaluating the singlet and doublet sequencing runs for clone overlap by aligning the sequencing runs and either scoring identity in at least 8 bases beyond the endonuclease recognition site which are identical in the doublet sequence with the singlet sequence or, alternatively, by scoring at least 16 cases beyond the endonuclease recognition site where the singlet sequence is consistent with either of the bases in the doublet sequence at that position, with less than 3 discordant positions.

36. A method according to claim 33, wherein a singlet and a triplet sequencing run in the same representation set from separate genomic clones are compared, said method further comprising:

evaluating whether the clones overlap by aligning the sequencing runs, considering only those positions in the triplet run where two or less

bases are read, and either scoring identity in at least 8 bases beyond the endonuclease recognition site which are identical in the triplet sequence with the singlet sequence or, alternatively, by scoring at least 16 cases beyond the endonuclease recognition site where the singlet sequence is consistent with either of the bases in the triplet sequence at that position, with less than 3 discordant positions.

37. A method according to claim 33, wherein a doublet and a doublet sequencing run in the same representation set from separate genomic clones are compared, said method further comprising:

evaluating whether the clones overlap by aligning the sequencing runs and scoring identity in at least 16 cases beyond the endonuclease recognition site which are either cases where either doublet sequence has an identical base which is consistent with one or the other of the two bases represented in the other doublet sequence, or cases where both doublet sequences have the same two bases at that position, with less than 3 discordant positions.

38. A method according to claim 33, wherein a doublet and a triplet sequencing run in the same representation set from separate genomic clones are compared, said method further comprising:

evaluating whether the clones overlap by aligning the sequencing runs, considering only those positions where two or less bases are read, and scoring identity in at least 16 cases beyond the endonuclease recognition site which are either cases where either doublet or triplet sequence has an identical base which is consistent with one or the other of the two bases represented in the other sequence, or cases where the doublet and triplet sequences have the same two bases at that position, with less than 3 discordant positions.

39. A method according to claim 33, wherein two sequencing runs from separate genomic clones in the same representation are compared with either run being a singlet, doublet, or triplet, said method further comprising:

evaluating whether the clones are likely not to overlap by aligning the sequencing runs and scoring discordance in at least 3 positions.

40. A method according to claim 1, wherein said combining clone overlap and sequence information comprises:

comparing sequence information in a second representation, present on clones which mark ends of contiguous portions of a first representation, to extend and overlap contigs between representations.

41. A method according to claim 1, wherein said combining clone overlap and sequence information comprises:

generating sequence information using a different restriction endonuclease representation on clones which mark ends of contiguous portions in a first representation.

42. A method according to claim 1, wherein said combining clone overlap and sequence information comprises:

using singlet sequences in the representations and end sequences for each clone to provide additional sequence information for aligning contiguous portions with the known databases for that organism.

43. A method according to claim 1, wherein said combining clone overlap and sequence information comprises:

obtaining unique singlet sequence information from overlapping doublet and triplet sequences, to provide additional sequence information for aligning contiguous portions with the known databases for that organism.

44. A method of identifying single nucleotide polymorphisms in genomic DNA comprising:

creating representations of the genomes of multiple individuals;

creating a representational library from the representation;

generating nucleic acid sequence information from individual clones of the representational library; and

analyzing the sequence information to identify single nucleotide polymorphisms among the multiple individuals.

45. A method according to claim 44, wherein said creating representations of the genomes of multiple individuals comprises:

subjecting the genomes of multiple individuals to a first restriction endonuclease under conditions effective to cleave DNA so that a first non-palindromic overhang is created in the genomes of multiple individuals;

subjecting the genomes of multiple individuals to a one of more second restriction endonuclease under conditions effective to cleave DNA so that a second overhang is created in the genomes of multiple individuals;

adding complementary linker adapters to the first and second overhangs in the presence of ligase, the first restriction endonuclease, and the one or more second restriction endonuclease; and

adding PCR primers to amplify fragments from the restriction endonuclease digest as a representation.

46. A method according to claim 45, wherein the first restriction endonuclease creates 2 base degenerate overhangs in the genomes of multiple individuals and 1 to 12 non-palindromic linker adapters, which contain single stranded overhangs of the formula NN/N'N' where NN/N'N' is selected from the group consisting of AA/TT, AC/GT, AG/CT, CA/TG, GA/TC, and GG/CC, are used.

47. A method according to claim 45, wherein the first restriction endonuclease creates 3 base degenerate overhangs in the genomes of multiple individuals and 1 to 16 non-palindromic complementary linker adapters, which contain single stranded overhangs of the formula NAA, NAC, NAG, NAT, NCA, NCC, NCG, NCT, NGA, NGC, NGG, NGT, NTA, NTC, NTG, and NTT, with N being any nucleotide, are used.

48. A method according to claim 45, wherein the first restriction endonuclease is selected from the group consisting of *Drd*I, *Bgl*I, *Dra*III, *Alw*NI,

*Pfl*MI, *San*DI, *Sex*AI, *Ppu*I, *Ava*II, *Eco*O109, *Bsu*36I, *Bsr*DI, *Bsg*I, *Bpm*I, *Sap*I, and an isoschizomer thereof and the one or more second restriction endonuclease is *Mae*II, *Msp*I, *Bfa*I, *Hha*I, *Hin*P1I, *Csp*6I, *Taq*I, *Mse*I, or an isoschizomer thereof.

49. A method according to claim 44, wherein said creating representations of the genomes of multiple individuals comprises;

subjecting the genomes of multiple individuals to a first restriction endonuclease under conditions effective to cleave DNA so that a palindromic overhang is created in the genomes of multiple individuals; and

adding complementary linker adapters to the overhangs in the presence of ligase and the first restriction endonuclease; and

adding PCR primers to amplify fragments from the restriction endonuclease digest as a representation.

50. A method according to claim 49, wherein the first restriction endonuclease is *Bam*HI, *Avr*II, *Nhe*I, *Spe*I, *Xba*I, *Kpn*I, *Sph*I, *Aat*II, *Age*I, *Xma*I, *Ngo*MI, *Bsp*EI, *Mlu*I, *Sac*II, *Bsi*WI, *Pst*I, *Apa*LI, or isoschizomers thereof.

51. A method according to claim 45, wherein PCR primers amplify fragments from the restriction endonuclease digest as a representation and a single linker-adapter primer is used to select fragments containing only one of the degenerate overhangs and the representation of the genome contains approximately 35,500 fragments.

52. A method according to claim 51, wherein a size selection of approximately 200 to 1,000 bp is applied prior to amplification, and the representation of the genome contains approximately 19,700 fragments.

53. A method according to claim 51, wherein a size selection of approximately 200 to 2,000 bp is applied prior to amplification, and the representation of the genome contains approximately 25,000 fragments.

54. A method according to claim 45, wherein PCR primers amplify fragments from the restriction endonuclease digest as a representation and more than one linker-adapter primer is used to select fragments containing some of the degenerate overhangs, a size selection of approximately 200 to 1,000 bp is applied prior to amplification, and the representation of the genome contains approximately 40,000 fragments.

55. A method according to claim 45, wherein PCR primers amplify fragments from the restriction endonuclease digest as a representation and more than one linker-adapter primer is used to select fragments containing some of the degenerate overhangs, a size selection of approximately 200 to 1,000 bp is applied prior to amplification, and the representation of the genome contains approximately 120,000 fragments.

56. A method according to claim 45, wherein PCR primers amplify fragments from the restriction endonuclease digest as a representation and a single linker-adapter primer is used to select fragments containing only one of the degenerate overhangs, a size selection of approximately 200 to 1,000 bp is applied prior to amplification, a PCR primer with one or two selective bases on the 3' end is used during the PCR amplification step, and the representation of the genome contains approximately 5,000 fragments.

57. A method according to claim 44, wherein a representational library is created from the representation and the linker-adapters used to generate the representation are methylated and PCR primers used to amplify the representation are unmethylated, such that the PCR amplified fragments may be cleaved in both primers to allow for directional cloning of fragments into a cloning vector.

58. A method for large scale detection of single nucleotide polymorphisms on a DNA array comprising:

creating a representation of the genome from a clinical sample;

providing a plurality of oligonucleotide probe sets, each set characterized by (a) a first oligonucleotide probe, having a target-specific portion and an addressable array-specific portion, and (b) a second oligonucleotide probe, having a target-specific portion and a detectable reporter label, wherein the oligonucleotide probes in a particular set are suitable for ligation together when hybridized adjacent to one another on a corresponding target nucleotide sequence, but have a mismatch which interferes with such ligation when hybridized to any other nucleotide sequence present in the representation of the sample;

providing a ligase,

blending the sample, the plurality of oligonucleotide probe sets, and the ligase to form a mixture;

subjecting the mixture to one or more ligase detection reaction cycles comprising a denaturation treatment, wherein any hybridized oligonucleotides are separated from the target nucleotide sequences, and a hybridization treatment, wherein the oligonucleotide probe sets hybridize at adjacent positions in a base-specific manner to their respective target nucleotide sequences, if present in the sample, and ligate to one another to form a ligated product sequence containing (a) the addressable array-specific portion, (b) the target-specific portions connected together, and (c) the detectable reporter label, and, wherein the oligonucleotide probe sets may hybridize to nucleotide sequences in the sample other than their respective target nucleotide sequences but do not ligate together due to a presence of one or more mismatches and individually separate during the denaturation treatment;

providing a support with different capture oligonucleotides immobilized at particular sites, wherein the capture oligonucleotides have nucleotide sequences complementary to the addressable array-specific portions;

contacting the mixture, after said subjecting, with the support under conditions effective to hybridize the addressable array-specific portions to the capture oligonucleotides in a base-specific manner, thereby capturing the addressable array-specific portions on the support at the site with the complementary capture oligonucleotide; and

detecting the reporter labels of ligated product sequences captured on the support at particular sites, thereby indicating the presence of single nucleotide polymorphisms.

59. A method according to claim 58, wherein the oligonucleotide probes in a set are suitable for ligation together at a ligation junction when hybridized adjacent to one another on a corresponding target nucleotide sequence due to perfect complementarity at the ligation junction, but, when the oligonucleotide probes in the set are hybridized to any other nucleotide sequence present in the sample, have a mismatch at a base at the ligation junction which interferes with such ligation.

60. A method according to claim 59, wherein the mismatch is at the 3' base at the ligation junction.

61. A method according to claim 58, wherein said creating a representation of the genome from a clinical sample comprises:

subjecting the clinical sample to a first restriction endonuclease under conditions effective to cleave DNA so that a first non-palindromic overhang is created in the clinical sample;

subjecting the clinical sample to a one of more second restriction endonuclease under conditions effective to cleave DNA so that a second overhang is created in the clinical sample;

adding complementary linker adapters to the first and second overhangs in the presence of ligase, the first restriction endonuclease, and the one or more second restriction endonuclease; and

adding PCR primers to amplify fragments from the restriction endonuclease digest as a representation.

62. A method according to claim 61, wherein the first restriction endonuclease creates 2 base degenerate overhangs in the clinical sample and 1 to 12 non-palindromic linker adapters, which contain single stranded overhangs of the

formula NN/N'N' where NN/N'N' is selected from the group consisting of AA/TT, AC/GT, AG/CT, CA/TG, GA/TC, and GG/CC, are used.

63. A method according to claim 61, wherein the first restriction endonuclease creates 3 base degenerate overhangs in the clinical sample and 1 to 16 non-palindromic complementary linker adapters, which contain single stranded overhangs of the formula NAA, NAC, NAG, NAT, NCA, NCC, NCG, NCT, NGA, NGC, NGG, NGT, NTA, NTC, NTG, and NTT, with N being any nucleotide, are used.

64. A method according to claim 61, wherein the first restriction endonuclease is selected from the group consisting of *Drd*I, *Bgl*I, *Dra*III, *Alw*NI, *Pfl*MI, *San*DI, *Sex*AI, *Ppu*I, *Ava*II, *Eco*O109, *Bsu*36I, *Bsr*DI, *Bsg*I, *Bpm*I, *Sap*I, and an isoschizomer thereof and the one or more second restriction endonuclease is *Mae*II, *Msp*I, *Bfa*I, *Hha*I, *Hin*P1I, *Csp*6I, *Taq*I, *Mse*I, or an isoschizomer thereof.

65. A method according to claim 58, wherein said creating representations of the genomes of a clinical sample comprises:

subjecting the clinical sample to a first restriction endonuclease under conditions effective to cleave DNA so that a palindromic overhang is created in the clinical sample;

adding complementary linker adapters to the overhangs in the presence of ligase and the first restriction endonuclease; and

adding PCR primers to amplify fragments from the restriction endonuclease digest as a representation.

66. A method according to claim 58, wherein the first restriction endonuclease is *Bam*HI, *Avr*II, *Nhe*I, *Spe*I, *Xba*I, *Kpn*I, *Sph*I, *Aat*II, *Age*I, *Xma*I, *Ngo*MI, *Bsp*EI, *Mlu*I, *Sac*II, *Bsi*WI, *Pst*I, *Apa*LI, or isoschizomers thereof.

67. A method according to claim 61, wherein PCR primers amplify fragments from the restriction endonuclease digest as a representation and a size

selection of approximately 200 to 2,000 bp is applied prior to amplification, improving the yield of fragments in the representation.

68. A method according to claim 61, wherein a single linker-adapter primer is used to select fragments containing only one of the degenerate overhangs and a PCR primer complementary to this linker adapter with one additional selective base on the 3' end is used during the PCR amplification step.

69. A method according to claim 61, wherein more than one linker-adapter primers are used to select fragments containing some of the degenerate overhangs and PCR primers complementary to the more than one linker adapter with one additional selective base on the 3' end are used.

70. A method according to claim 61, wherein PCR primers amplify fragments from the restriction endonuclease digest as a representation and a single linker-adapter primer is used to select fragments containing only one of the degenerate overhangs and PCR primers complementary to this linker adapter with one additional selective base on the 3' end are used.

71. A method according to claim 61, wherein PCR primers amplify fragments from the restriction endonuclease digest as a representation and a single linker-adapter primer is used to select fragments containing only one of the degenerate overhangs and PCR primers complementary to this linker adapter with two additional selective bases on the 3' end is used during the PCR amplification step.

72. A method according to claim 61, wherein PCR primers amplify fragments from the restriction endonuclease digest as a representation and a single linker-adapter primer is used to select fragments containing only one of the degenerate overhangs and a PCR primer complementary to this linker adapter with two additional selective bases on the 3' end is used during the PCR amplification step.

73. A method according to claim 58, wherein said plurality of oligonucleotide probe sets comprises:

(a) a first oligonucleotide probe, having a target-specific portion complementary to a first allele and a first addressable array-specific portion, (b) a second oligonucleotide probe, having a target-specific portion complementary to a second allele and a second addressable array-specific portion and (c) a third oligonucleotide probe, having a target-specific portion and a detectable reporter label, wherein the first and third oligonucleotide probes set are suitable for ligation together when hybridized adjacent to one another on a corresponding first allele target nucleotide sequence, wherein the second and third oligonucleotide probes set are suitable for ligation together when hybridized adjacent to one another on a corresponding second allele target nucleotide sequence, but each set has a mismatch which interferes with such ligation when hybridized to any other nucleotide sequence present in the representation of the sample and, wherein the reporter labels of ligation product sequences captured to the support at particular sites during said detecting where the presence of reporter label at the complement of the first addressable array-specific portion indicates the presence of the first allele, while presence of reporter label at the complement of the second addressable array-specific portion indicates the presence of the second allele, for each set, thereby indicating allele differences.

74. A method according to claim 73, wherein the oligonucleotide probes in a set are suitable for ligation together at a ligation junction when hybridized adjacent to one another on a corresponding target nucleotide sequence due to perfect complementarity at the ligation junction, but, when the oligonucleotide probes in the set are hybridized to any other nucleotide sequence present in the sample, have a mismatch at a base at the ligation junction which interferes with such ligation.

75. A method according to claim 73, wherein the mismatch is at the 3' base at the ligation junction.

76. A method according to claim 73, wherein the first and second alleles differ by a single nucleotide.

77. A method according to claim 73, wherein said method is used to quantify an allele imbalance between first and second alleles and the different capture oligonucleotides immobilized at particular sites are substantially the same for both the first allele target nucleotide sequence and the second allele target nucleotide sequence, wherein the oligonucleotide probe sets have either of two reporter labels which can be detected and distinguished independently so that ligation product sequences for the first allele target nucleotide sequence and the second allele target nucleotide sequence are captured on the support with the ratio of the first reporter label to the second reporter label at the complement of the first addressable array-specific portion divided by the ratio of the first reporter label to the second reporter label at the complement of the second addressable array-specific portion reflecting an initial allele ratio for each test and normal allele position and the relative imbalance of the first and second alleles in a test sample is determined by dividing the initial allele ratio for the test sample by the initial allele ratio for a normal sample, whereby (1) a ratio of > 1 indicates that the first allele is in that number-fold greater in quantity than the second allele, (2) a ratio of < 1 indicates that the second allele is in the inverse number-fold greater in quantity than the first allele, and (3) a ratio of about 1 indicates the first and second allele are present in about the same quantity.

78. A method according to claim 77, wherein said method is for quantifying loss of heterozygosity (LOH) or gene amplification in a tumor sample containing up to 50% stromal contamination by comparing allele imbalance at a tumor gene locus with allele balance at a control gene locus among a tumor and normal sample from the same individual and the different capture oligonucleotides immobilized at particular sites are substantially the same for both the first allele target nucleotide sequence and the second allele target nucleotide sequence, the two alleles being heterozygous at both the tumor gene locus and the control gene locus with the ratio of the first reporter label to the second reporter label at the complement of the first addressable array-specific portion for the tumor gene locus divided by the ratio of the first reporter label to the second reporter label at the complement of the first addressable array-specific portion for the control gene locus reflecting an initial tumor

to control first allele ratio, wherein for both test and normal samples where the ratio of the first reporter label to the second reporter label at the complement of the second addressable array-specific portion for the tumor gene locus divided by the ratio of the first reporter label to the second reporter label at the complement of the second addressable array-specific portion for the control gene locus reflects an initial tumor to control second allele ratio and a presence of gene amplification or LOH of the first and second tumor alleles in the tumor sample is determined by dividing the initial tumor to control level for a given allele ratio for the tumor sample by the initial tumor to control level for a given allele ratio for the normal sample where (1) a ratio of > 2 for a first tumor gene allele indicates the first tumor gene allele is amplified in the tumor sample, compared with the normal sample, (2) a ratio of > 2 for a second tumor gene allele indicates the second tumor gene allele is amplified in the tumor sample, compared with the normal sample, (3) a ratio of < 0.5 for a first tumor gene allele shows that the first tumor gene allele underwent LOH in the tumor sample, compared with the normal sample, (4) a ratio of < 0.5 for a second tumor gene allele shows that the second tumor gene allele underwent LOH in the tumor sample, compared with the normal sample, and (5) a ratio of about 1 indicates a given tumor allele did not undergo LOH or amplification, compared with the normal sample.

79. A method according claim according to claim 73, wherein the method is utilized for quantifying an allele imbalance between a test sample and a normal sample with each set characterized by both first and second oligonucleotide probes, a percentage of each have a second distinct detectable reporter label, wherein the two reporter labels may be detected and distinguished independently such that the ratio of the first reporter label to the second reporter label at the complement of the first addressable array-specific portion divided by the ratio of the first reporter label to the second reporter label at the complement of the second addressable array-specific portion reflects an initial allele ratio for each test and normal allele position and a relative imbalance of the first and second alleles in the test sample is determined by dividing the initial allele ratio for the test sample by the initial allele ratio for the normal sample, wherein (1) a ratio of > 1 indicates that the first allele is in that number-fold greater in quantity than the second allele, (2) a ratio of < 1 indicates that

the second allele is in the inverse number-fold greater in quantity than the first allele, and (3) a ratio of about 1 indicates that the first and second allele are present in about the same quantity, indicating there is no allele imbalance compared with the normal sample.

80. A method according to claim 79, wherein said method is carried out for quantifying loss of heterozygosity (LOH) or gene amplification in a tumor sample containing up to 50% stromal contamination by comparing allele imbalance at a tumor gene locus with allele balance at a control gene locus among a tumor and normal sample from the same individual with the two alleles being heterozygous at both the tumor gene locus and the control gene locus and the ratio of the first reporter label to the second reporter label at the complement of the first addressable array-specific portion for the tumor gene locus divided by the ratio of the first reporter label to the second reporter label at the complement of the first addressable array-specific portion for the control gene locus reflecting an initial tumor to control first allele ratio, such that for both test and normal samples, the ratio of the first reporter label to the second reporter label at the complement of the second addressable array-specific portion for the tumor gene locus divided by the ratio of the first reporter label to the second reporter label at the complement of the second addressable array-specific portion for the control gene locus reflects an initial tumor to control second allele ratio and the presence of gene amplification or LOH of the first and second tumor alleles in the tumor sample is determined by dividing the initial tumor to control for a given allele ratio for the tumor sample by the initial tumor to control for a given allele ratio for the normal sample, wherein (1) a ratio of > 2 for a first tumor gene allele indicates the first tumor gene allele is amplified in the tumor sample, compared with the normal sample, (2) a ratio of > 2 for a second tumor gene allele indicates the second tumor gene allele is amplified in the tumor sample, compared with the normal sample, (3) a ratio of < 0.5 for a first tumor gene allele indicates the first tumor gene allele underwent LOH in the tumor sample, compared with the normal sample, (4) a ratio of < 0.5 for a second tumor gene allele indicates the second tumor gene allele underwent LOH in the tumor sample, compared with the

normal sample, and (5) a ratio of about 1 indicates a given tumor allele did not undergo LOH or amplification, compared with the normal sample.

81. A method according to claim 58, wherein said providing a plurality of oligonucleotide probe sets with each set characterized by (a) a first oligonucleotide probe, having a target-specific portion complementary to a first allele and a first detectable reporter label, (b) a second oligonucleotide probe, having a target-specific portion complementary to a second allele and a second distinct detectable reporter label and (c) a third oligonucleotide probe, having a target-specific portion and a addressable array-specific portion, wherein the first and third oligonucleotide probes set are suitable for ligation together when hybridized adjacent to one another on a corresponding first allele target nucleotide sequence, wherein the second and third oligonucleotide probes set are suitable for ligation together when hybridized adjacent to one another on a corresponding second allele target nucleotide sequence, but each set has a mismatch which interferes with such ligation when hybridized to any other nucleotide sequence present in the representation of the sample with the two reporter labels being detected and distinguished independently such that detection of the first reporter label at the complement of the addressable array-specific portion indicates a presence of the first allele, while detection of the second reporter label at the complement of the addressable array-specific portion indicates a presence of the second allele, for each set.

82. A method according to claim 81, wherein the mismatch is at a 3' base at the ligation junction.

83. A method according to claim 81, wherein the first and second alleles differ by a single nucleotide.

84. A method for according to claim 81, wherein said method is used to quantify an allele imbalance between first and second alleles and the different capture oligonucleotides immobilized at particular sites are substantially the same for both the first allele target nucleotide sequence and the second allele target nucleotide

sequence, wherein the oligonucleotide probe sets have either of two reporter labels which can be detected and distinguished independently so that ligation product sequences for the first allele target nucleotide sequence and the second allele target nucleotide sequence are captured on the support at particular sites with the ratio of the first reporter label to the second reporter label at the complement of the first addressable array-specific portion divided by the ratio of the first reporter label to the second reporter label at the complement of the second addressable array-specific portion reflecting an initial allele ratio for each test and normal allele position and the relative imbalance of the first and second alleles in the test sample is determined by dividing the initial allele ratio for the test sample by the initial allele ratio for the normal sample, whereby (1) a ratio of > 1 indicates that the first allele is in that number-fold greater quantity over the second allele, (2) a ratio of < 1 indicates that the second allele is in the inverse number-fold greater quantity over the first allele, and (3) a ratio of about 1 determines the first and second allele are present in about the same quantity.

85. A method according to claim 81, wherein said method is for quantifying loss of heterozygosity (LOH) or gene amplification in a tumor sample containing up to 50% stromal contamination by comparing allele imbalance at a tumor gene locus with allele balance at a control gene locus among a tumor and normal sample from the same individual and the different capture oligonucleotides immobilized at particular sites are substantially the same for both the first allele target nucleotide sequence and the second allele target nucleotide sequence, the two alleles being heterozygous at both the tumor gene locus and the control gene locus with the ratio of the first reporter label to the second reporter label at the complement of the first addressable array-specific portion for the tumor gene locus divided by the ratio of the first reporter label to the second reporter label at the complement of the first addressable array-specific portion for the control gene locus reflecting an initial tumor to control first allele ratio, wherein for both test and normal sample where the ratio of the first reporter label to the second reporter label at the complement of the second addressable array-specific portion for the tumor gene locus divided by the ratio of the first reporter label to the second reporter label at the complement of the second

addressable array-specific portion for the control gene locus reflects an initial tumor to control second allele ratio and a presence of gene amplification or LOH of the first and second tumor alleles in the tumor sample is determined by dividing the initial tumor to control level for a given allele ratio for the tumor sample by the initial tumor to control level for a given allele ratio for the normal sample where (1) a ratio of > 2 for a first tumor gene allele indicates the first tumor gene allele is amplified in the tumor sample, compared with the normal sample, (2) a ratio of > 2 for a second tumor gene allele indicates the second tumor gene allele is amplified in the tumor sample, compared with the normal sample, (3) a ratio of < 0.5 for a first tumor gene allele determines the first tumor gene allele underwent LOH in the tumor sample, compared with the normal sample, (4) a ratio of < 0.5 for a second tumor gene allele determines the second tumor gene allele underwent LOH in the tumor sample, compared with the normal sample, (5) a ratio of about 1 determines a given tumor allele did not undergo LOH or amplification, compared with the normal sample.

86. A method according claim according to claim 81, wherein the method is utilized for quantifying an allele imbalance between a test sample and a normal sample with each set characterized by both first and second oligonucleotide probes, a percentage of each have a second distinct detectable reporter label, wherein the two reporter labels may be detected and distinguished independently such that the ratio of the first reporter label to the second reporter label at the complement of the first addressable array-specific portion divided by the ratio of the first reporter label to the second reporter label at the complement of the second addressable array-specific portion reflects an initial allele ratio for each test and normal allele position and the relative imbalance of the first and second alleles in the test sample is determined by dividing the initial allele ratio for the test sample by the initial allele ratio for the normal sample, wherein (1) a ratio of > 1 indicates that the first allele is in that number-fold greater quantity over the second allele, (2) a ratio of < 1 indicates that the second allele is in the inverse number-fold greater quantity over the first allele, and (3) a ratio of about 1 indicates that the first and second allele are present in about the same quantity, indicating there is no allele imbalance compared with the normal sample.

87. A method according to claim 81, wherein said method is carried out for quantifying loss of heterozygosity (LOH) or gene amplification in a tumor sample containing up to 50% stromal contamination by comparing allele imbalance at a tumor gene locus with allele balance at a control gene locus among a tumor and normal sample from the same individual with the two alleles being heterozygous at both the tumor gene locus and the control gene locus and the ratio of the first reporter label to the second reporter label at the complement of the first addressable array-specific portion for the tumor gene locus divided by the ratio of the first reporter label to the second reporter label at the complement of the first addressable array-specific portion for the control gene locus reflecting an initial tumor to control first allele ratio, such that for both test and normal sample, the ratio of the first reporter label to the second reporter label at the complement of the second addressable array-specific portion for the tumor gene locus divided by the ratio of the first reporter label to the second reporter label at the complement of the second addressable array-specific portion for the control gene locus reflects an initial tumor to control second allele ratio and the presence of gene amplification or LOH of the first and second tumor alleles in the tumor sample is determined by dividing the initial tumor to control for a given allele ratio for the tumor sample by the initial tumor to control for a given allele ratio for the normal sample, wherein (1) a ratio of > 2 for a first tumor gene allele indicates the first tumor gene allele is amplified in the tumor sample, compared with the normal sample, (2) a ratio of > 2 for a second tumor gene allele indicates the second tumor gene allele is amplified in the tumor sample, compared with the normal sample, (3) a ratio of < 0.5 for a first tumor gene allele indicates the first tumor gene allele underwent LOH in the tumor sample, compared with the normal sample, (4) a ratio of < 0.5 for a second tumor gene allele indicates the second tumor gene allele underwent LOH in the tumor sample, compared with the normal sample, and (5) a ratio of about 1 indicates a given tumor allele did not undergo LOH or amplification, compared with the normal sample.

88. A method to sequence directly from a PCR amplified nucleic acid molecule without primer interference comprising:

amplifying a nucleic acid molecule using PCR primers containing alternative nucleoside bases under conditions effective to produce PCR amplification products and

cleaving the PCR primers both incorporated and unincorporated in the PCR amplification products under conditions which leave the PCR amplification products intact.

89. A method according to claim 88, wherein the PCR primers contain dUTP and starting primers and incorporated primers are cleaved using uracil-N-glycosylase (ung) prior to DNA sequencing.

90. A method according to claim 88, wherein the PCR primers contain ribonucleosides and starting primers and incorporated primers are cleaved with a base (0.1N NaOH) followed by neutralization with a buffer prior to DNA sequencing.

Sequencing *DrdI* islands in random plasmid or cosmid clones

1. PCR amplify fragment from random clone of a genomic DNA library. Cut with *DrdI* in the presence of linkers and T4 ligase. Linker for *DrdI* site is phosphorylated and contains a 3' AA overhang. Biochemical selection assures that most AA sites contain linkers. (Separate reactions are performed for linkers containing the other non-palindromic 3' overhangs).

2. Inactivate T4 ligase and restriction endonucleases at 95°C for 5 min. Add longer sequencing primer which contains a 3' AA end, and perform a cycle-sequencing reaction. If sequence information is difficult to interpret, additional selectivity can be achieved by preforming four separate sequencing reactions using sequencing primers containing 3' ends of AAA, AAC, AAG, and AAT respectively.

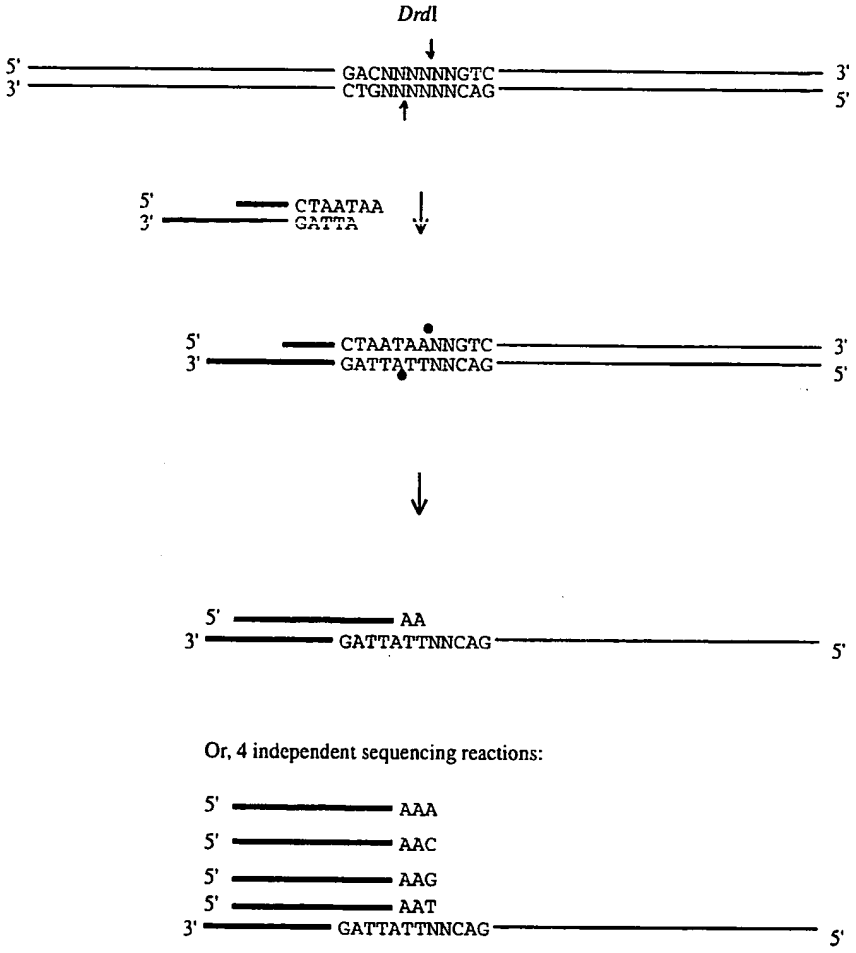


FIG. 1

Scheme 1 for sequencing restriction endonuclease generated representations

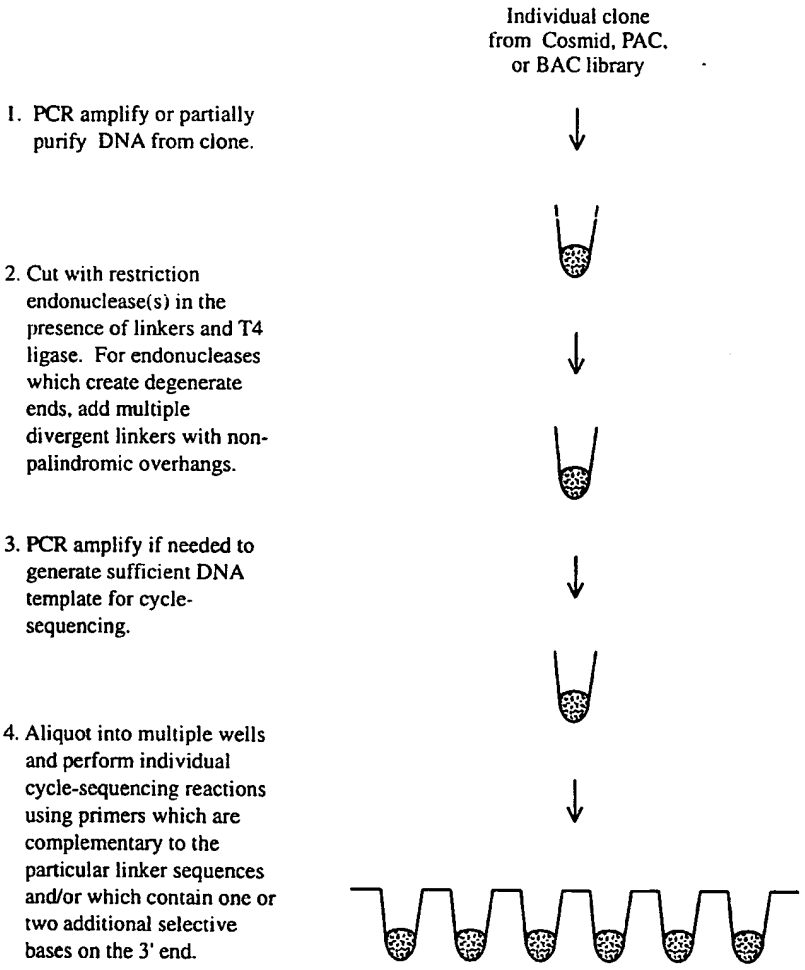


FIG. 2

Scheme 2 for sequencing restriction endonuclease generated representations

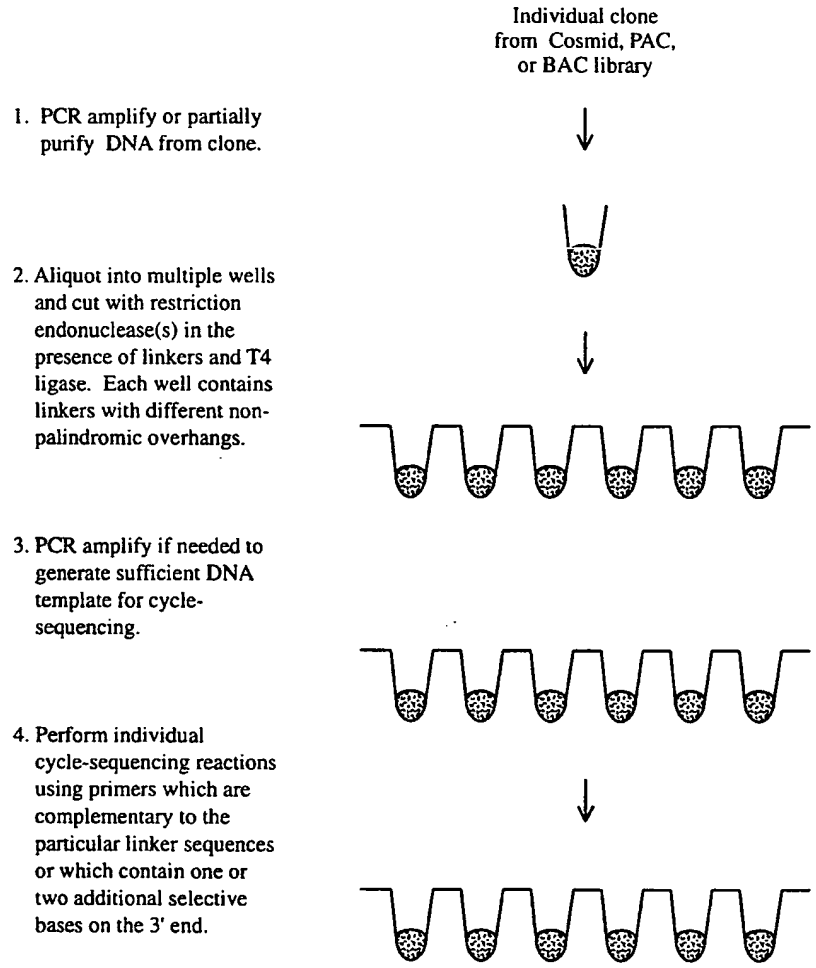


FIG. 3

DNA sequencing directly from PCR amplified DNA without primer interference

1. PCR amplify using oligonucleotides containing ribose U replacing dT, add dNTPs and *Taq* polymerase.
2. Add 0.1 N NaOH and heat to 95°C for 5 min to destroy unused primers.
3. Neutralize, dilute into two new wells. Anneal forward and reverse primers in separate reactions to run fluorescent dideoxy-sequencing reactions.

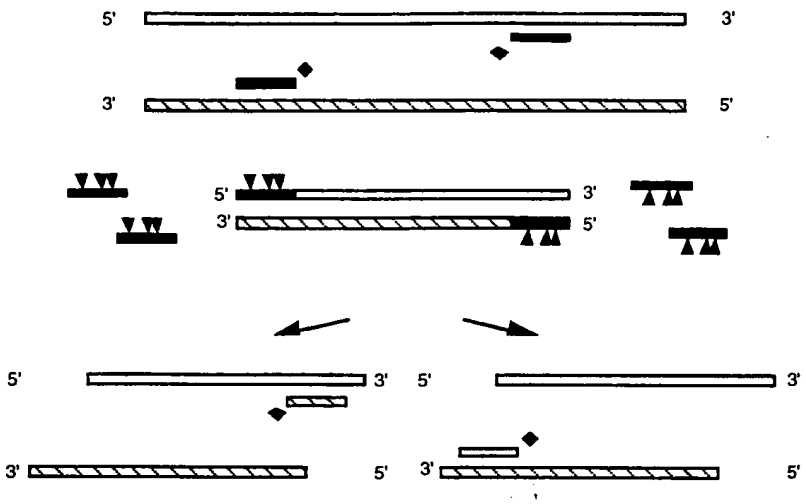


FIG. 4

Sequencing *DrdI* islands in random BAC clones

1. Cut BAC DNA with *MspI* and *DrdI* in the presence of linkers and T4 ligase. Linker for *DrdI* site is phosphorylated and contains a 3' AA overhang. Linker for *MspI* site is not phosphorylated, and contains a bubble. Biochemical selection assures that most sites contain linkers.

2. Inactivate T4 ligase and restriction endonucleases at 95°C for 5 min. PCR amplify using primers containing ribose U replacing dT, dNTPs, and *Taq* polymerase. Primer specific to the *DrdI* site linker will extend through bubble of *MspI* site linker. This allows the primer specific to the *MspI* site linker to amplify the *DrdI*-*MspI* fragment. *MspI*-*MspI* fragments will not amplify since they contain bubbles on both ends.

3. Add 0.1N NaOH and heat to 95 °C for 5 min to destroy unused primers.

4. Neutralize and dilute. Anneal sequencing primer to the *DrdI* site linker and perform a cycle-sequencing reaction. (A separate reaction may be performed using a primer annealing to the *MspI* site linker).

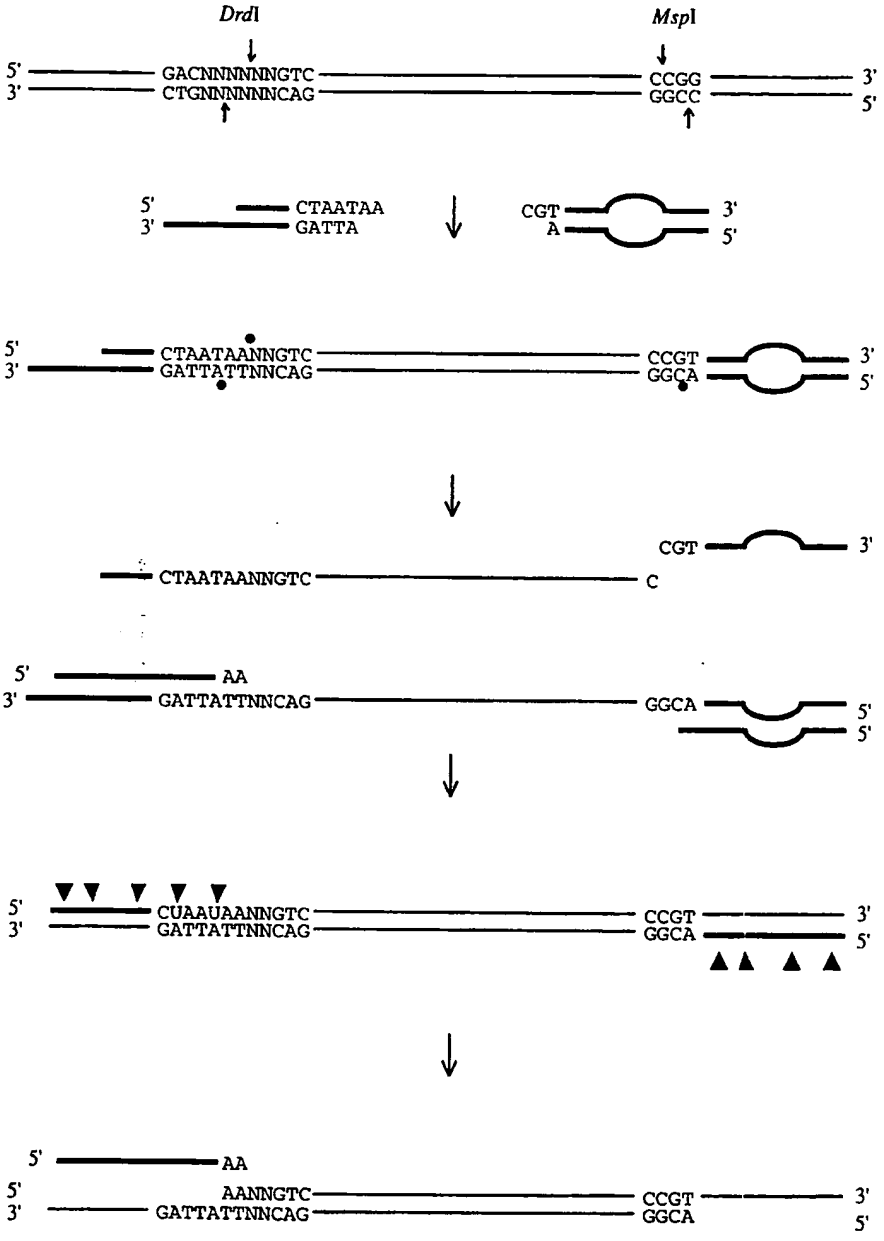


FIG. 5

Sequencing *DrdI* islands in random BAC clones

1. Cut BAC DNA with *DrdI*, *MspI* and *TaqI* in the presence of linkers and T4 ligase. Linker for *DrdI* site is phosphorylated and contains a 3' AA overhang. Linker for *MspI/TaqI* site is phosphorylated, 3' blocked and contains a bubble. Biochemical selection assures that most sites contain linkers.

2. Inactivate T4 ligase and restriction endonucleases at 95°C for 5 min. PCR amplify using primers containing ribose U replacing dT, dNTPs, and *Taq* polymerase. Primer specific to the *DrdI* site linker will extend through bubble of *MspI* site linker. This allows the primer specific to the *MspI* site linker to amplify the *DrdI-MspI* fragment. Other fragments will not amplify since they contain bubbles on both ends.

3. Add 0.1N NaOH and heat to 95 °C for 5 min to destroy unused primers.

4. Neutralize and dilute. Anneal sequencing primer to the *DrdI* site linker and perform a cycle-sequencing reaction. (A separate reaction may be performed using a primer annealing to the *MspI/TaqI* site linker).

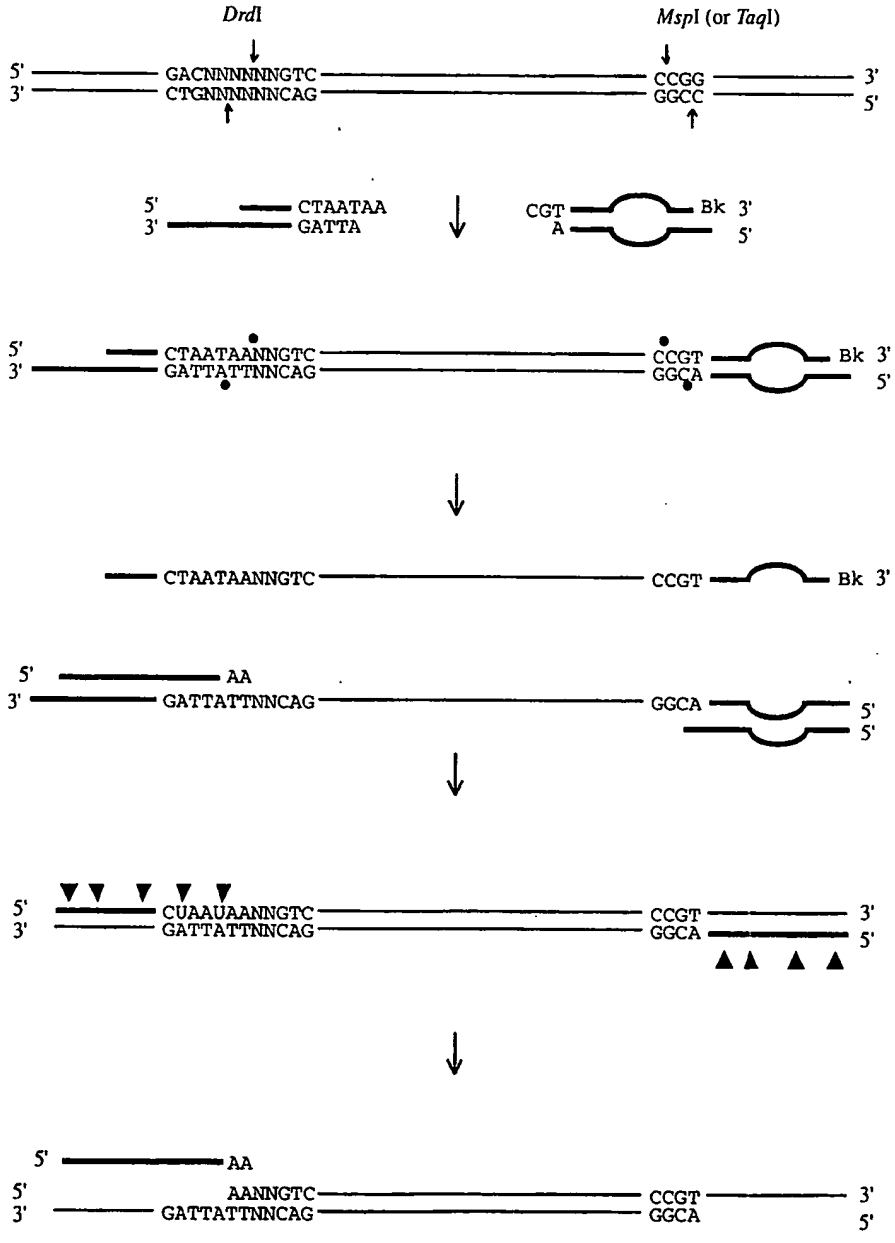
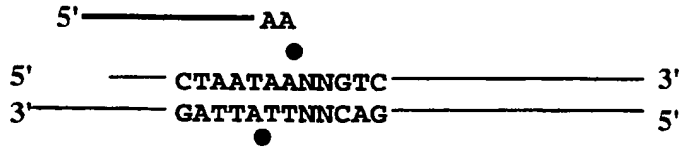


FIG. 6

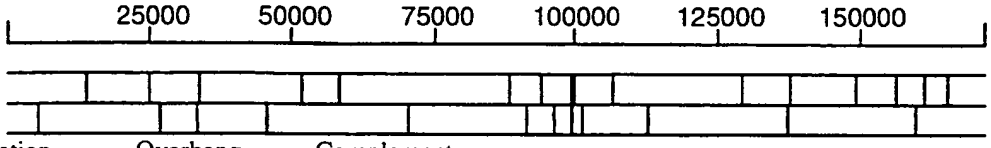
Three degrees of specificity in amplifying a *Drd*I representation.



1. Ligation of the top strand requires perfect complementarity at the 3' side of the junction (50-fold specificity).
2. Ligation of the bottom strand requires perfect complementarity at the 3' side of the junction (50-fold specificity).
3. Extension of polymerase off the sequencing primer is most efficient if the 3' base is perfectly matched (10 to 100-fold specificity).

FIG. 7

RG253B13, 7q31 Met Oncogene
12 *Drd*I and 16 *Bgl*ISites in 171,905 bp



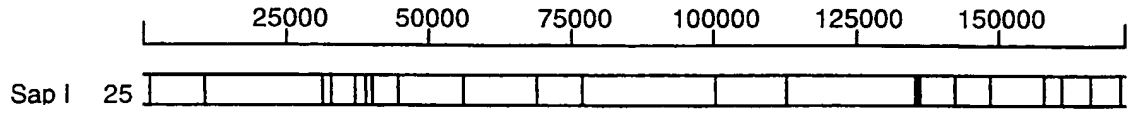
*Drd*I#	Location	Overhang	Complement
1.	5,379	GG*#	CC*#
2.	26,865	GT#	AC#
3.	33,300	GG*#	CC*#
4.	45,528	AT^	AT^
5.	70,522	AT^	AT^
6.	91,675	TC@	GA@
7.	96,500	CA@	TG@
8.	99,622	CT#	AG#
9.	101,434	TT*#	AA*#
10.	113,042	AC#	GT#
11.	137,171	TT*#	AA*#
12.	159,679	AG#	CT#

*Bgl*I#	Location	Overhang	Complement
1.	13,833	TGT*X	ACA*X
2.	25,115	ACA*X	TGT*X
3.	33,890	GAAX	TTC@
4.	51,623	TGT*X	ACA*X
5.	58,308	CTA*#	TAG#
6.	88,316	TTA*#	TAAX
7.	94,134	GGG*#	CCC*#
8.	99,463	ACA*X	TGT*X
9.	100,045	ACC*#	GGT*X
10.	106,613	CCA*X	TGG*#
11.	129,192	TGT*X	ACA*X
12.	137,747	TCT@	AGA*#
13.	149,246	TGT*X	ACA*X
14.	156,577	TTT@	AAAX
15.	161,461	CGA*#	TCG@
16.	165,697	CTG@	CAG#

		*Drd*I	*Bgl*I
Unique sites, per 40 kb (singlet).		(1.4)	(3.3)
*Same last 2 bases of 3' overhang, per 40 kb (doublet).	(1.0)	(4.3)	
^Palindromic overhang, not used.		2	
@Same last 2 bases of 3' overhang within Bac used exactly once (singlet).		2	5
#Same last 2 bases of 3' overhang within Bac used exactly twice (doublet).		4	5
XSame last 2 bases of 3' overhang within BAC used more than twice.		0	3

FIG. 8

RG253B13, 7q31 Met Oncogene
25 *Sap*I Sites in 171,905 bp

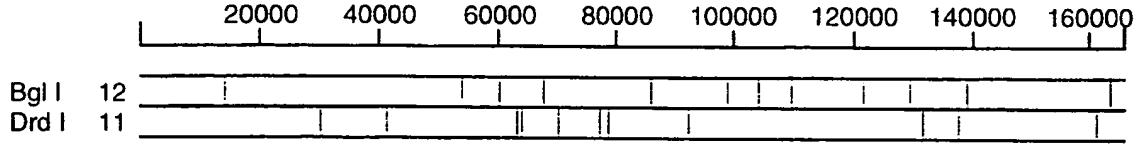


*Sap*I#	Location	*Sap*I Overhang	Ligated Complement
1.	1,198	CTA	TAG#
2.	1,456	AGG	CCT#
3.	10,943	GCT	AGC^x
4.	10,955	GCT	ACG@
5.	11,041	CAA	TTG@
6.	31,031	AAT	ATT@
7.	32,599	GAT	ATC#
8.	37,053	AGA	TCT#
9.	38,931	GGG	CCC^x
10.	39,877	ATC	GAT#
11.	44,325	CTT	AAG#
12.	56,040	ACA	TGT^x
13.	68,850	ACC	GGT^x
14.	76,930	GTG	CAC#
15.	100,250GGG	CCC^x	
16.	112,850GAT	ATC#	
17.	135,473ACA	TGT^x	
18.	135,608GGA	TCC^x	
19.	136,239TTG	CAA@	
20.	142,243GCC	GGC^x	
21.	148,475GCG	CGC^x	
22.	157,978TCT	AGA@	
23.	160,833ACC	GGT^x	
24.	166,153ATT	AAT#	
25.	171,460GTT	AAC#	

	*Sap*I
@Same last 2 bases of 3' overhang within BAC used exactly once(singlet).	5
#Same last 2 bases of 3' overhang within BAC used exactly twice (doublet).	10
xSame last 2 bases of 3' overhang within BAC used more than twice.	3

FIG. 9

RG363E19, 7q3.1 HMG gene
11 *DrdI* and 12 *BglI*Sites in 165,608 bp



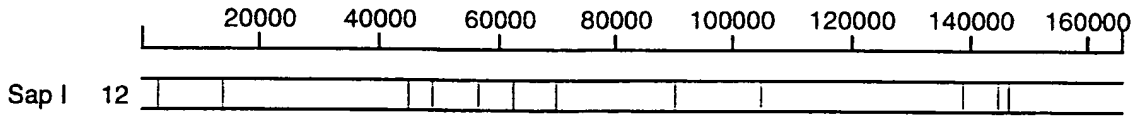
DrdI#	Location	Overhang	Complement
1.	30,500	CT*#	AG*#
2.	41,442	GG#	CC#
3.	63,326	AG*#	CT*#
4.	64,189	TT@	AA@
5.	70,300	GT@	AC@
6.	77,512	CA*x	TG*x
7.	78,858	TG*x	CA*x
8.	92,723	TG*x	CA*x
9.	132,104	GA@	TC@
10.	137,827	CC#	GG#
11.	161,478	AT^	AT^

BglI#	Location	Overhang	Complement
1.	14,666	GAG#	CTC#
2.	54,284	AGA*x	TCT*x
3.	60,389	AGA*x	TCT*x
4.	67,808	CCT*x	AGG*x
5.	86,331	TGG*x	CCA#
6.	99,283	CTC#	GAG#
7.	104,281	GTT#	AAC@
8.	109,938	CGG*x	CCG@
9.	122,096	GGG*x	CCC@
10.	129,631	TGT@	ACA#
11.	139,404	AAA@	TTT#
12.	163,611	TCTx	AGAx

	DrdI	*BglI*
Unique sites, per 40 kb (singlet).	(1.2)	(3.9)
*Same last 2 bases of 3' overhang, per 40 kb (doublet).	(1.2)	(2.0)
^Palindromic overhang, not used.	1	

FIG. 10

RG363E19, 7q3.1 HMG gene
12 *Sap*I Sites in 165,608 bp

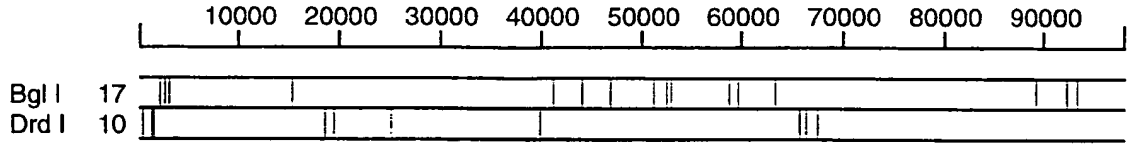


*Sap*I#	Location	*Sap*I Overhang	Ligated Complement
1.	3,048	ACA	TGT[@]
2.	14,192	CGG	CCG[@]
3.	45,137	CTA	TAG[x]
4.	49,039	TAC	GTA[#]
5.	56,731	CCT	AGG[@]
6.	62,838	TAA	TTA[#]
7.	70,117	TGG	CCA[@]
8.	90,393	AAA	TTT[x]
9.	104,917	CTT	AAG[x]
10.	138,863	CTG	CAG[x]
11.	144,649	AAA	TTT[x]
12.	146,805	AAA	TTT[x]

	*Sap*I
[@]Same last 2 bases of 3' overhang within BAC used exactly once (singlet).	4
[#]Same last 2 bases of 3' overhang within BAC used exactly twice (doublet).	1
[x]Same last 2 bases of 3' overhang within BAC used more than twice.	2

FIG. 11

RG364P16, 7q31 Pendrin gene
10 *DrdI* and 17 *BglI* Sites in 97,943 bp



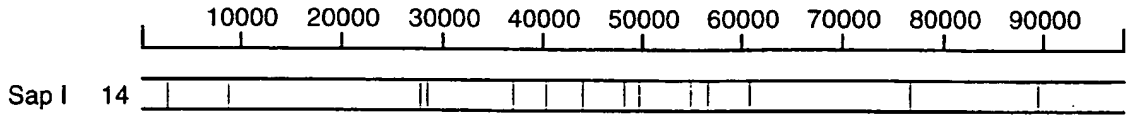
DrdI#	Location	Overhang	Complement
1.	620	CC[@]	GG[@]
2.	1,478	CT[*x]	AG[*x]
3.	1,697	CT[*x]	AG[*x]
4.	18,677	AG[*x]	CT[*x]
5.	19,514	TT[@]	AA[@]
6.	25,223	GA[@]	TC[@]
7.	39,952	AA[*#]	TT[*#]
8.	65,731	AA[*#]	TT[*#]
9.	66,419	TA[^]	TA[^]
10.	67,512	GC[^]	GC[^]

BglI#	Location	Overhang	Complement
1.	2,239	GGC[*x]	GCC[*#]
2.	2,779	AAC[x]	GTT[#]
3.	3,237	GGC[*x]	GCC[*#]
4.	15,292	ATG[*x]	CAT[x]
5.	41,224	CAC[*x]	GTG[*x]
6.	44,028	GCA[*x]	TGC[x]
7.	46,962	AGG[*x]	CCT[*x]
8.	51,342	AGT[*#]	ACT[*x]
9.	52,497	TTA[#]	TAA[#]
10.	53,002	AAT[x]	ATT[#]
11.	53,027	TGG[*x]	CCA[*x]
12.	58,872	CAC[*x]	GTG[*x]
13.	59,766	CCT[*x]	AGG[*x]
14.	63,339	TGG[*x]	CCA[*x]
15.	89,301	TGT[*#]	ACA[*x]
16.	92,307	ATA[#]	TAT[x]
17.	93,558	TTC[@]	GAA[#]

	DrdI	*BglI*
Unique sites, per 40 kb (singlet).	(1.3)	(5.0)
[*]Same last 2 bases of 3' overhang, per 40 kb (doublet).	(2.1)	(9.2)
[^]Palindromic overhang, not used.	2	
[@]Same last 2 bases of 3' overhang within Bac used exactly once (singlet).	3	1
[#]Same last 2 bases of 3' overhang within Bac used exactly twice (doublet).	1	5
[x]Same last 2 bases of 3' overhang within BAC used more than twice.	1	7

FIG. 12

RG364P16, 7q31 Pendrin gene
14 *Sap*I Sites in 97,943 bp

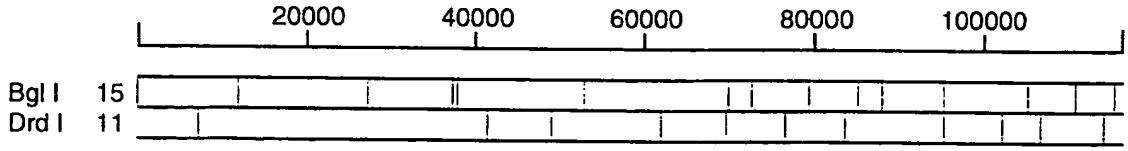


*Sap*I#	Location	*Sap*I Overhang	Ligated Complement
1.	2,731	CTA	TAG[#]
2.	8,819	ATA	TAT[@]
3.	27,714	CAG	CTG[X]
4.	28,452	TCT	AGA[@]
5.	37,174	GAA	TTC[@]
6.	40,339	GTT	AAC[@]
7.	44,149	CAC	GTG[X]
8.	48,133	AAC	GTT[@]
9.	49,746	CTT	AAG[#]
10.	55,020	TTT	AAA[#]
11.	56,593	CAG	CTG[X]
12.	60,911	AGA	TCT[@]
13.	76,747	TTA	TAA[#]
14.	89,658	TGA	TCA[@]

	*Sap*I
[@]Same last 2 bases of 3' overhang within BAC used exactly once (singlet).	7
[#]Same last 2 bases of 3' overhang within BAC used exactly twice (doublet).	2
[X]Same last 2 bases of 3' overhang within BAC used more than twice.	1

FIG. 13

GS056H18, 7q31 alpha2(I) collagen
11 *Drd*I and 15 *Bgl*ISites in 116,466 bp



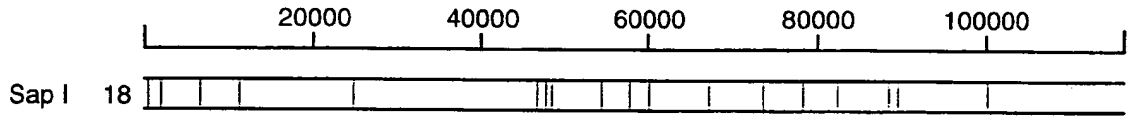
*Drd*I#	Location	Overhang	Complement
1.	7,281	AA*#	TT*#
2.	41,553	AA*#	TT*#
3.	49,116	TG#	CA#
4.	61,875	GT*#	AC*#
5.	69,731	AC*#	GT*#
6.	76,744	AG@	CT@
7.	83,697	GG@	CC@
8.	95,410	TA^	TA^
9.	102,312	TC*#	GA*#
10.	107,014	TC*#	GA*#
11.	114,581	CA#	TG#

*Bgl*I#	Location	Overhang	Complement
1.	26	CAG*x	CTG*x
2.	12,014	TTA#	TAA*#
3.	27,316	CTG*x	CAG*x
4.	37,513	AAA*@	TTT@
5.	37,810	GTA*#	TAC#
6.	52,919	CTG*x	CAG*x
7.	70,083	ACA*x	TGT*x
8.	72,753	ACA*x	TGT*x
9.	79,674	CGA#	TCG*#
10.	85,304	GCG*#	CGC@
11.	88,200	GTC*#	GAC#
12.	95,350	GAA#	TTC*#
13.	105,353	ACA*x	TGT*x
14.	111,096	CCC*#	GGG@
15.	115,757	TCC*#	GGA#

	*Drd*I	*Bgl*I
Unique sites, per 40 kb (singlet).	(1.4)	(3.1)
*Same last 2 bases of 3' overhang, per 40 kb (doublet).	(2.1)	(7.2)
^Palindromic overhang, not used.	1	
@Same last 2 bases of 3' overhang within Bac used exactly once (singlet).	2	4
#Same last 2 bases of 3' overhang within Bac used exactly twice (doublet).	4	7
xSame last 2 bases of 3' overhang within BAC used more than twice.	0	3

FIG. 14

GS056H18, 7q31 alpha2(I) collagen
18 *Sap*I Sites in 116,466 bp



*Sap*I#	Location	*Sap*I Overhang	Ligated Complement
1.	676	AAA	TTT[x]
2.	2,235	CTC	GAG[x]
3.	6,921	CTG	CAG[x]
4.	11,596	ACC	GGT[#]
5.	24,903	GCT	AGC[#]
6.	46,819	AAA	TTT[x]
7.	47,742	TCC	GGA[#]
8.	48,563	ATT	AAT[@]
9.	54,507	TCT	AGA[#]
10.	57,797	ACT	AGT[#]
11.	60,140	TAC	GTA[@]
12.	67,461	AAG	CTT[x]
13.	73,821	AAT	ATT[x]
14.	78,670	CTG	CAG[x]
15.	82,755	CCT	AGG[@]
16.	88,654	AGT	ACT[@]
17.	89,773	GCA	TGC[#]
18.	100,380	CTC	GAG[x]

	*Sap*I
[@]Same last 2 bases of 3' overhang within BAC used exactly once(singlet).	4
[#]Same last 2 bases of 3' overhang within BAC used exactly twice (doublet).	3
[x]Same last 2 bases of 3' overhang within BAC used more than twice.	2

FIG. 15

Sequencing *BglI* islands in random BAC clones

1. Cut BAC DNA with *MspI* and *BglI* in the presence of linkers and T4 ligase. Linker for *BglI* site is phosphorylated and ends in 3' NAC overhang. Linker for *MspI* site is not phosphorylated, and contains a bubble. Biochemical selection assures that most sites contain linkers.

2. Inactivate T4 ligase and restriction endonucleases at 95°C for 5 min. PCR amplify using primers containing ribose U replacing dT, dNTPs, and *Taq* polymerase. Add 0.1N NaOH and heat to 95 °C for 5 min to destroy unused primers.

3. Neutralize and dilute. Anneal sequencing primer to the *BglI* site linker and perform a cycle-sequencing reaction. (A separate reaction may be performed using a primer annealing to the *MspI* site linker).

4. A separate linker ligation reaction is performed on the second half of the *BglI* site using a phosphorylated primer which ends in a 3' NTA seqence.

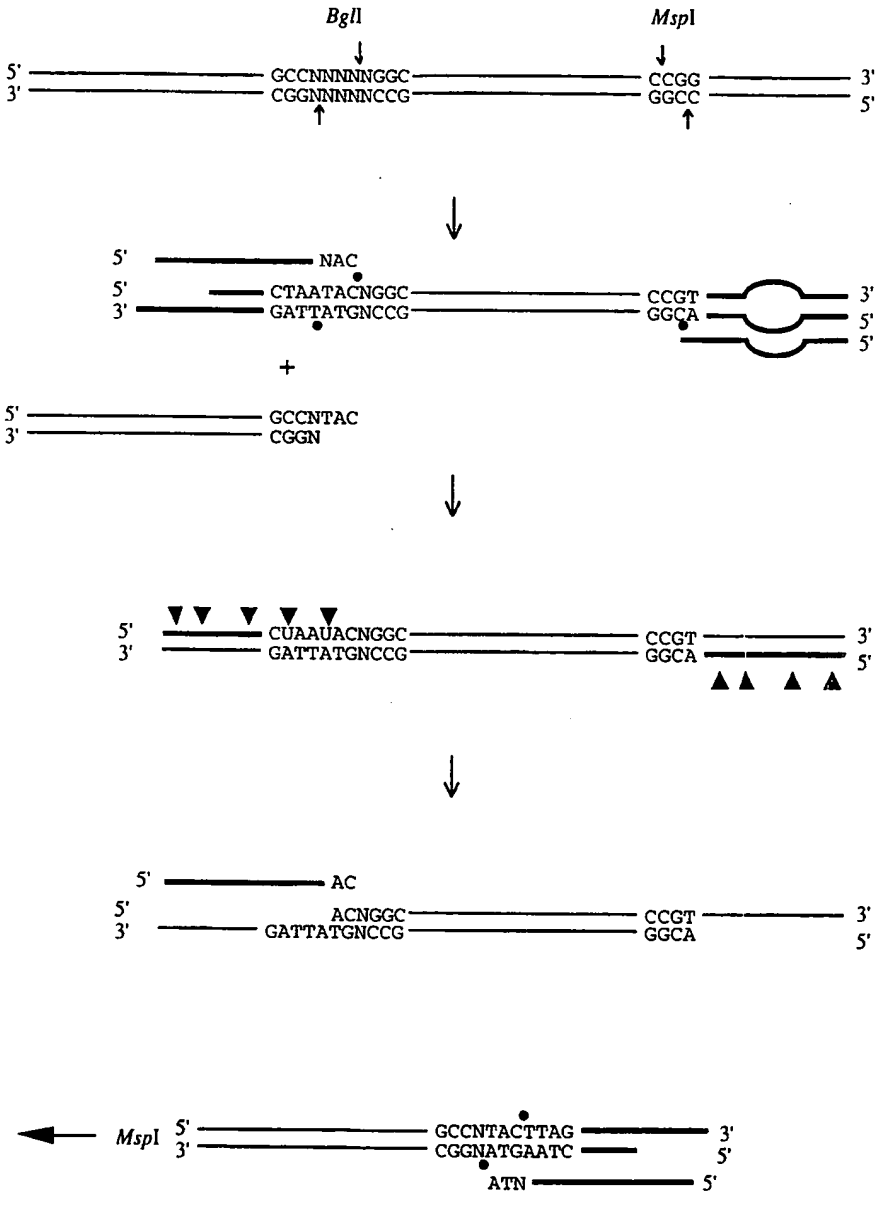


FIG. 16

Sequencing *BglI* islands in random BAC clones

1. Cut BAC DNA with *MspI* and *BglI* in the presence of linkers and T4 ligase. Linker for *BglI* site is phosphorylated and ends in 3' ACN overhang. Linker for *MspI* site is not phosphorylated, and contains a bubble. Biochemical selection assures that most sites contain linkers.

2. Inactivate T4 ligase and restriction endonucleases at 95°C for 5 min. PCR amplify using primers containing ribose U replacing dT, dNTPs, and *Taq* polymerase. Primer specific to the *BglI* site linker will extend through bubble of *MspI* site linker. This allows the primer specific to the *MspI* site linker to amplify the *DrdI*-*MspI* fragment. *MspI*-*MspI* fragments will not amplify since they contain bubbles on both ends.

3. Add 0.1N NaOH and heat to 95 °C for 5 min to destroy unused primers.

4. Neutralize and dilute. Anneal sequencing primer to the *BglI* site linker and perform a cycle-sequencing reaction. (A separate reaction may be performed using a primer annealing to the *MspI* site linker).

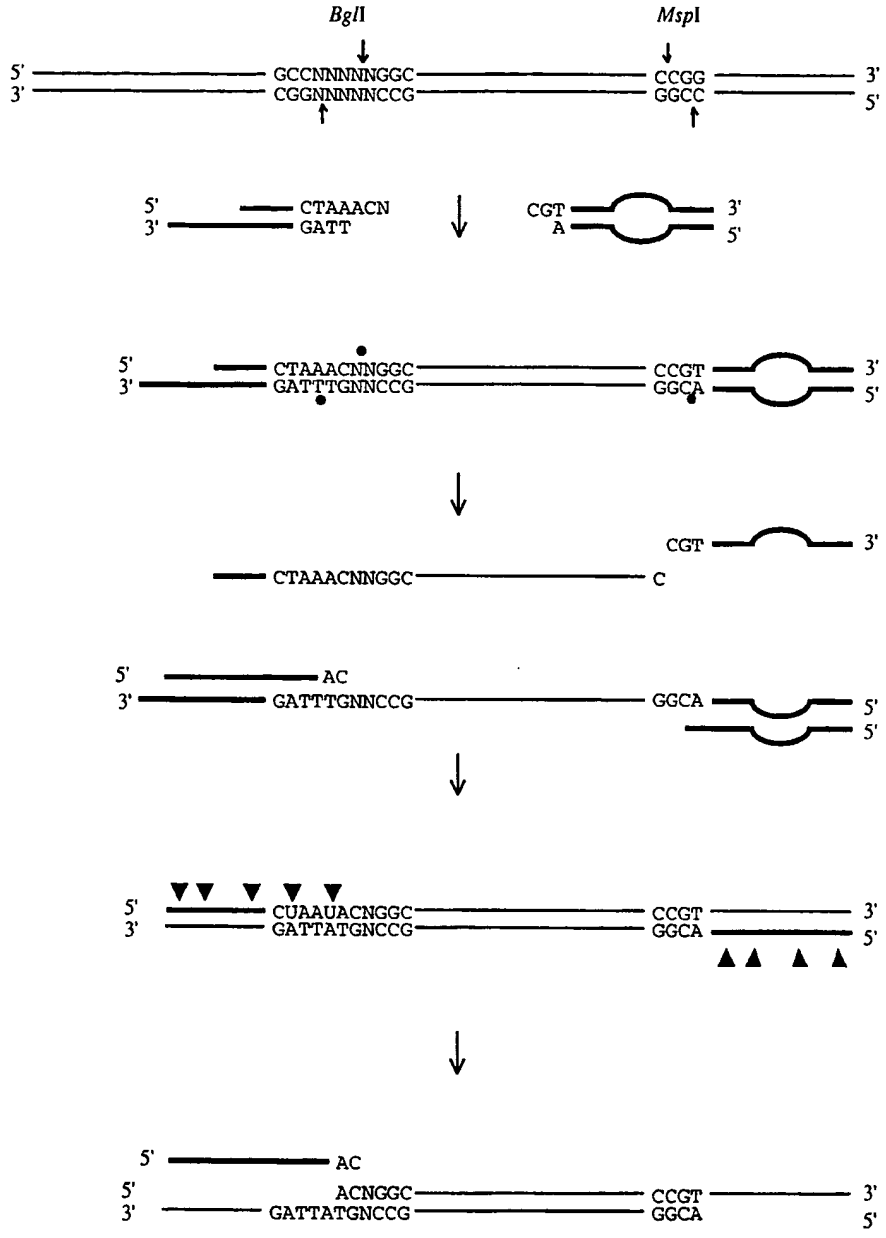


FIG. 16A

Sequencing *SapI* islands in random BAC clones

1. Cut BAC DNA with *MspI* and *SapI* in the presence of linkers and T4 ligase. Linker for *SapI* site is phosphorylated and ends in 5' NAC overhang. Linker for *MspI* site is not phosphorylated, and contains a bubble. Biochemical selection assures that most sites contain linkers.

2. Inactivate T4 ligase and restriction endonucleases at 95°C for 5 min. PCR amplify using primers containing ribose U replacing dT, dNTPs, and *Taq* polymerase. Add 0.1N NaOH and heat to 95 °C for 5 min to destroy unused primers.

3. Neutralize and dilute. Anneal sequencing primer to the *SapI* site linker and perform a cycle-sequencing reaction. (A separate reaction may be performed using a primer annealing to the *MspI* site linker).

4. A separate linker ligation reaction is performed on the second half of the *SapI* site using a phosphorylated primer which ends in a 3' NTA seqence. However, this reforms the *SapI* site, and thus the linker is cleaved off preventing substantial DNA amplification.

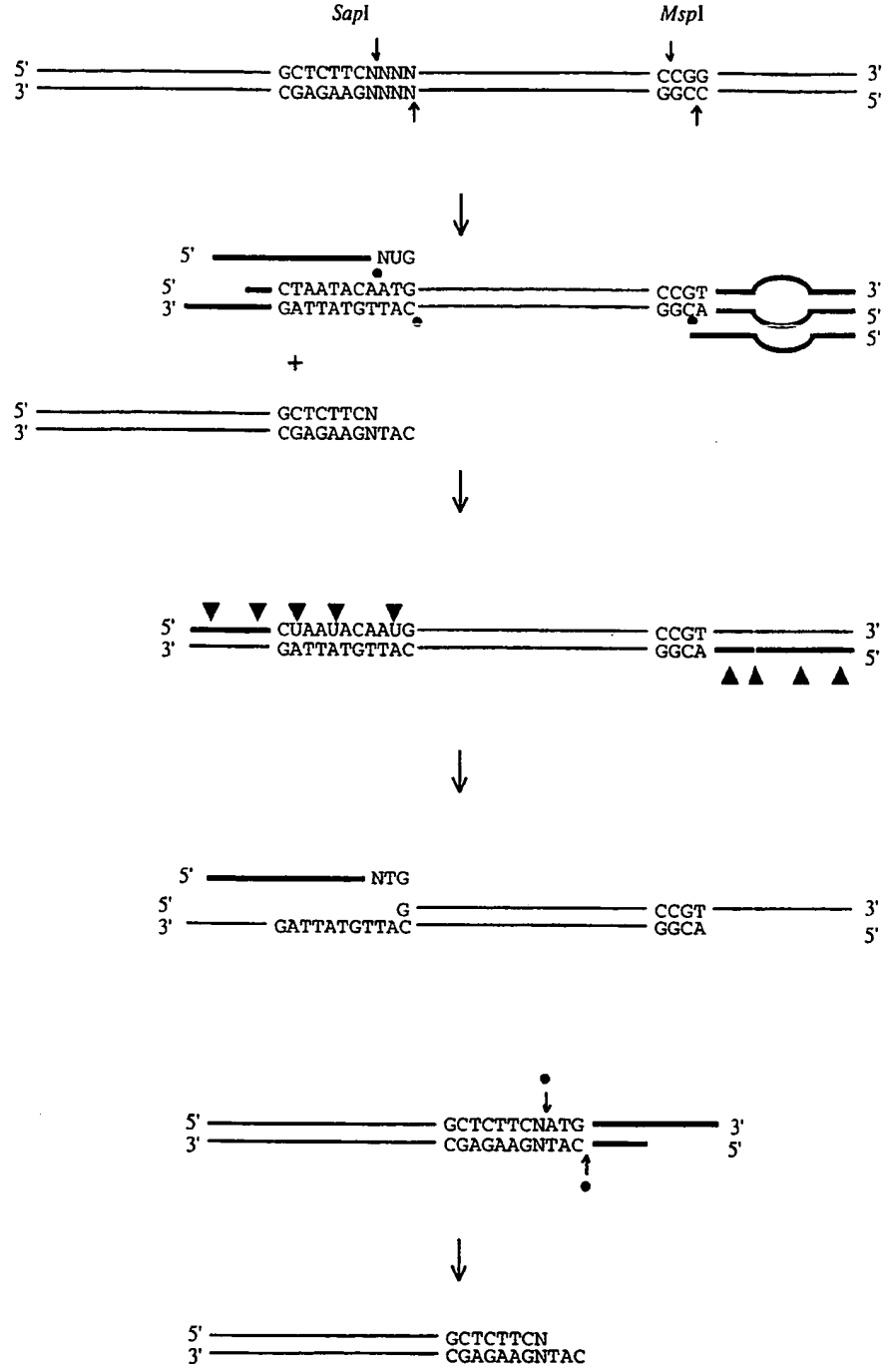
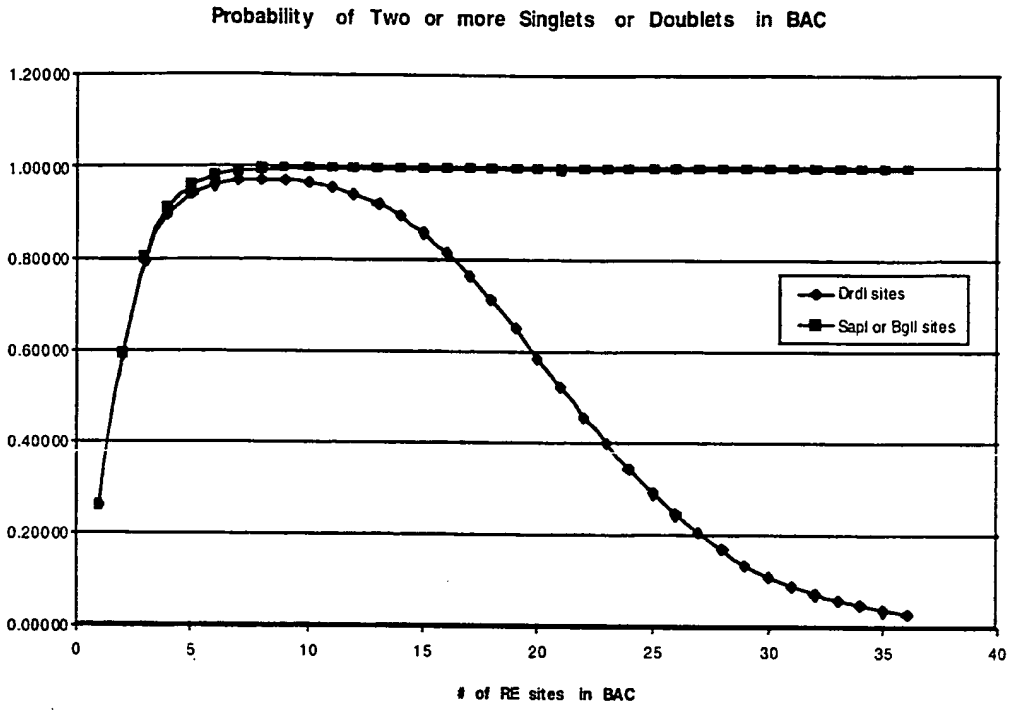


FIG. 17


Probability of Two or more Singlets or Doublets in BAC
1.20000
1.00000
0.80000
0.60000
0.40000
0.20000
0.00000
0
5
10
15
20
25
30
35
40
# of RE sites in BAC
DrdI sites
SapI or BglI sites

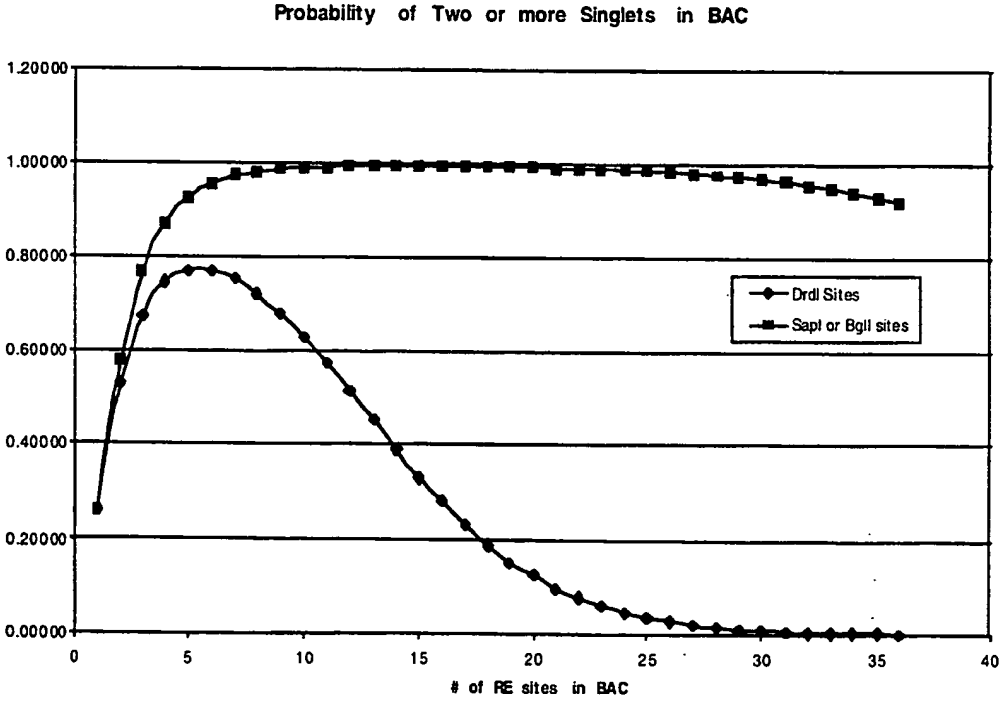

Probability of Two or more Singlets in BAC
1.20000
1.00000
0.80000
0.60000
0.40000
0.20000
0.00000
0
5
10
15
20
25
30
35
40
# of RE sites in BAC
DrdI Sites
SapI or BglI sites

FIG. 17A

Alignment of BAC sequences generated from *DrdI* sites:

```
1. TCGTCCTCAGGAACTGAAGCTATATAATCAGTTAAGTCCCTGCTTCTGATCTCTTCTGATTTTCTTCTAAGAAGAGAATA
2. GTGTCAAGTAAAGAAGTACAGCAGATAAGTAAAACGGAAAAAAATAATGAAAGAATTACAAAGGAAGACTAAGGAAAGAG
3. AAGTCTACAATCAAGAGGCCAACTGATTCCATGTCTGGTGAGGGTCTATTTCCTGGTGCATAGATGGCTCCTTCTCACTG
4. TAGTCCTCAATTTCACCATGGATTAAATAACAGAACACAGAGTTACTGTGAGACTTGTGGTAGAAAATCTTTAATTCATT
5. GTGTCATCTAGCTATAAATCTAAAGATAATAATAAAATTGGAAAGATTTTCATCAGATAGACTTTTAACACCAAGCTTGA
```

Concordant sequences: Doublet to singlet.

```
1. TCGTCCTCAGGAACTGAAGCTATATAATCAGTTAAGTCCCTGCTTCTGATCTCTTCTGATTTTCTTCTAAGAAGAGAATA
2. GTGTCAAGTAAAGAAGTACAGCAGATAAGTAAAACGGAAAAAAATAATGAAAGAATTACAAAGGAAGACTAAGGAAAGAG
1. TCGTCCTCAGGAACTGAAGCTATATAATCAGTTAAGTCCCTGCTTCTGATCTCTTCTGATTTTCTTCTAAGAAGAGAATA
```

Concordant sequences: Doublet to Doublet.

```
1. TCGTCCTCAGGAACTGAAGCTATATAATCAGTTAAGTCCCTGCTTCTGATCTCTTCTGATTTTCTTCTAAGAAGAGAATA
2. GTGTCAAGTAAAGAAGTACAGCAGATAAGTAAAACGGAAAAAAATAATGAAAGAATTACAAAGGAAGACTAAGGAAAGAG
3. AAGTCTACAATCAAGAGGCCAACTGATTCCATGTCTGGTGAGGGTCTATTTCCTGGTGCATAGATGGCTCCTTCTCACTG
2. GTGTCAAGTAAAGAAGTACAGCAGATAAGTAAAACGGAAAAAAATAATGAAAGAATTACAAAGGAAGACTAAGGAAAGAG
```

Concordant sequences: Doublet to Triplet.

```
1. TCGTCCTCAGGAACTGAAGCTATATAATCAGTTAAGTCCCTGCTTCTGATCTCTTCTGATTTTCTTCTAAGAAGAGAATA
2. GTGTCAAGTAAAGAAGTACAGCAGATAAGTAAAACGGAAAAAAATAATGAAAGAATTACAAAGGAAGACTAAGGAAAGAG
3. AAGTCTACAATCAAGAGGCCAACTGATTCCATGTCTGGTGAGGGTCTATTTCCTGGTGCATAGATGGCTCCTTCTCACTG
2. GTGTCAAGTAAAGAAGTACAGCAGATAAGTAAAACGGAAAAAAATAATGAAAGAATTACAAAGGAAGACTAAGGAAAGAG
4. TAGTCCTCAATTTCACCATGGATTAAATAACAGAACACAGAGTTACTGTGAGACTTGTGGTAGAAAATCTTTAATTCATT
```

Discordant sequences: Doublet to singlet.

```
1. TCGTCCTCAGGAACTGAAGCTATATAATCAGTTAAGTCCCTGCTTCTGATCTCTTCTGATTTTCTTCTAAGAAGAGAATA
2. GTGTCAAGTAAAGAAGTACAGCAGATAAGTAAAACGGAAAAAAATAATGAAAGAATTACAAAGGAAGACTAAGGAAAGAG
      X    XX    XXX  X XXX X  X  XX X XXX  XX   XX XX  XX        X X XXXXXXXXX X
3. AAGTCTACAATCAAGAGGCCAACTGATTCCATGTCTGGTGAGGGTCTATTTCCTGGTGCATAGATGGCTCCTTCTCACTG
```

Discordant sequences: Doublet to Doublet.

```
1. TCGTCCTCAGGAACTGAAGCTATATAATCAGTTAAGTCCCTGCTTCTGATCTCTTCTGATTTTCTTCTAAGAAGAGAATA
2. GTGTCAAGTAAAGAAGTACAGCAGATAAGTAAAACGGAAAAAAATAATGAAAGAATTACAAAGGAAGACTAAGGAAAGAG
           XX    Xx      X        X X  X  x    X  x               X        xX XXx
3. AAGTCTACAATCAAGAGGCCAACTGATTCCATGTCTGGTGAGGGTCTATTTCCTGGTGCATAGATGGCTCCTTCTCACTG
4. TAGTCCTCAATTTCACCATGGATTAAATAACAGAACACAGAGTTACTGTGAGACTTGTGGTAGAAAATCTTTAATTCATT
```

Discordant sequences: Doublet to Triplet.

```
1. TCGTCCTCAGGAACTGAAGCTATATAATCAGTTAAGTCCCTGCTTCTGATCTCTTCTGATTTTCTTCTAAGAAGAGAATA
2. GTGTCAAGTAAAGAAGTACAGCAGATAAGTAAAACGGAAAAAAATAATGAAAGAATTACAAAGGAAGACTAAGGAAAGAG
            X   X                  X   X       X               x        xX XXx
3. AAGTCTACAATCAAGAGGCCAACTGATTCCATGTCTGGTGAGGGTCTATTTCCTGGTGCATAGATGGCTCCTTCTCACTG
4. TAGTCCTCAATTTCACCATGGATTAAATAACAGAACACAGAGTTACTGTGAGACTTGTGGTAGAAAATCTTTAATTCATT
5. GTGTCATCTAGCTATAAATCTAAAGATAATAATAAAATTGGAAAGATTTTCATCAGATAGACTTTTAACACCAAGCTTGA
```

FIG. 18

DrdI/MseI Fragments in approximately 2 MB of human DNA

(BACs anakyzed: RG253B13, RG013N12, RG300C03, RG022J17, RG067E13, RG011J21, RG022C01, RG043K06, RG343P13, RG205G13, O68P20, H_133K23, RG363E19, RG364P16, GS056H18, RG083J23, RG103H13, and RG118D07)

For AA overhangs (30 Fragments)

DrdI#	Location	Overhang	Complement	Nearest *MseI*	Fragment Length
9.	101,440		AA*(T)	100753	687
8.	125,589		AA*	124941	648
8.	65,737	AA*(C)		66359	622
2.	41,548	AA*(C)		41918	370
3.	21,755	AA*		22080	325
11.	148,484	AA*		148770	286
15.	180,054		AA*	179781	273
1.	7,287	AA*(A)		7551	264
4.	64,195		AA*	63964	231
2.	16192		AA*	16002	190
5.	19,520		AA*	19354	166
7.	112,864		AA*	112716	148
9.	67,981	AA*(A)		68102	121
10.	76,325	AA*(C)		76443	118
6.	73,322	AA*		73424	102
10.	158,579		AA*	158499	80
1.	9,941		AA*(C)	9867	74
8.	65,625		AA*	65554	71
6.	45,326		AA*	45263	63
14.	168,400		AA*	168352	48
7.	39,958	AA*(C)		40005	47
2.	27,073		AA*(A)	27027	46
8.	144,712	AA*(A)		144750	38
3.	30,987	AA*		31013	26
10.	114962	AA*		114986	24
4.	89309		AA*	89290	19
1.	4518	AA*		4532	14
11.	137,177		AA*(A)	137176	1
12.	165,140		AA*	165139	1
9.	86,690		AA*	86689	1

For AC overhangs (14 Fragments)

DrdI#	Location	Overhang	Complement	Nearest *MseI*	Fragment Length
4.	61,881		AC*	61424	457
5.	70,306		AC*	69996	400

FIG. 19

5.	51333	AC*		51712	379
2.	17,346		AC*	17135	211
2.	26,871		AC*	26668	203
2.	16,508	AC*		16703	195
4.	45929	AC*		46051	132
6.	104,064		AC*	103955	109
8.	80,512		AC*	80423	89
9.	113,009		AC*	112938	71
6.	100,564		AC*	100500	64
5.	69,737	AC*		69789	52
10.	113,048	AC*		113095	47
5.	89,050	AC*		89180	30

For AG overhangs (18 Fragments)

*Drd*I#	Location	Overhang	Complement	Nearest *Mse*I	Fragment Length
7.	124,720		AG*	123644	1076
8.	99,628		AG*	99513	546
7.	55,076		AG*	54728	348
11.	146,074	AG*		146412	338
3.	63,332	AG*		63546	214
2.	1,484		AG*	1273	211
1.	30,506		AG*	30700	194
4.	51345	AG*		51500	155
12.	159,685	AG*		159827	142
3.	1,703		AG*	1593	110
5.	26,574		AG*	26478	96
9.	125,495	AG*		125587	92
9.	84,646		AG*	84587	59
6.	76,750	AG*		76794	44
11.	137111		AG*	137072	39
5.	71871	AG*		71907	36
4.	18,683	AG*		18707	24
2.	27,400	AG*		27409	9

For CA overhangs (28 Fragments)

*Drd*I#	Location	Overhang	Complement	Nearest *Mse*I	Fragment Length
1.	11,050		CA*(T)	10453	597
5.	40,727	CA*(G)		41277	550
8.	92,729		CA*(G)	92225	504
4.	28263		CA*	27859	404
7.	96,506	CA*(A)		96800	294
7.	68476	CA*		68753	277

FIG. 19 (cont.)

3.	40,167		CA*(T)	39891	276
7.	104,893	CA*(G)		105141	248
12.	174,759		CA*(G)	174553	206
3.	24,762	CA*		24967	205
7.	78,864		CA*(T)	78672	192
3.	27,738	CA*(A)		27922	184
11.	114,587	CA*(G)		114739	152
4.	25,393	CA*(G)		25529	136
1.	1797		CA*(T)	1663	134
7.	56,328		CA*(A)	56194	134
5.	47,359		CA*(T)	47234	125
3.	49,122		CA*(G)	48998	124
11.	92,418	CA*(T)		92512	94
7.	142,867		CA*(G)	142773	94
12.	98,198	CA*(A)		98284	86
6.	60,501		CA*(T)	60424	77
8.	83,536	CA*(A)		83598	62
6.	77,518	CA*		77578	60
7.	41,602	CA*(T)		41644	42
9.	149,703	CA*(A)		149735	32
10.	128,190		CA*(G)	128168	22
5.	40,370		CA*(G)	40357	13

For GA overhangs (15 Fragments)

DrdI#	Location	Overhang	Complement	Nearest *MseI*	Fragment Length
10.	138,792		GA*	138206	586
10.	107,020		GA*	106698	322
8.	105,928		GA*	105714	214
9.	132,110	GA*		132317	207
6.	25,229	GA*		25384	155

Figure 19 (cont.)

1.	4,328		GA*	4225	103
4.	29,833	GA*		29929	96
13.	166,309	GA*		166386	77
4.	66,836		GA*	66763	73
8.	139,856		GA*	139797	59
9.	102,318		GA*	102277	41
5.	97330		GA*	97292	38
6.	91,681		GA*	91645	36
11.	153,548	GA*		153569	21
14.	169,979	GA*		169996	17

FIG. 19 (cont.)

For GG overhangs (14 Fragments)

*Drd*I#	Location	Overhang	Complement	Nearest *Mse*I	Fragment Length
3.	33,306	GG*		34241	935
3.	43,961	GG*		44471	510
2.	41,448	GG*		41745	297
7.	83,703	GG*		83957	254
13.	180,666		GG*	180498	168
2.	19,383		GG*	19227	156
10.	137,833		GG*	137722	111
5.	89,627		GG*	89570	57
9.	129,058		GG*	129003	55
9.	74,360	GG*		74409	49
12.	154,063		GG*	154021	42
1.	5,385	GG*		5417	32
1.	626		GG	596	30
6.	49,989	GG*		50001	12

FIG. 19 (cont.)

DrdI/MspI/TaqI Fragments in approximately 2 MB of human DNA

(RG253B13, RG013N12, RG300C03, RG022J17, RG067E13, RG011J21, RG022C01, RG043K06, RG343P13, RG205G13, O68P20, H_133K23, RG363E19, RG364P16, GS056H18, RG083J23, RG103H13, and RG118D07)

For AA overhangs (28 Fragments)

*Drd*I#	Location	Overhang	Complement	Nearest *Msp*I	Nearest *Taq*I	Fragment Length
14.	168,400		AA*	DrdI(157,688)	162,381	6,019
10.	158,579		AA*	151,605	153,001	5,578
2.	41,548	AA*(C)			46,609	5,061
1.	9,941		AA*(C)	296	6,494	3,447
7.	39,958	AA*(C)		43,295	45,578	3,337
7.	112,864		AA*	110,256	DrdI(104,064)	2,608
10.	114962	AA*		117286	120674	2324
9.	86,690		AA*	82,301	84,647	2,043
3.	21,755	AA*		27,904	23,795	2,040
9.	67,981	AA*(A)		71,232	69,660	1,679
10.	76,325	AA*(C)		79,607	77,651	1,326
8.	65,625		AA*	63,673	64,515	1,110
1.	4518	AA*		5549	5792	1031
4.	89309		AA*	88376	86730	933
11.	137,177		AA*(A)	135,890	136,580	597
3.	30,987	AA*		31,504	DrdI(32,405)	517
15.	180,054		AA*	179562	176427	492
8.	125,589		AA*	DrdI(124,720)	125,163	426
5.	73,322	AA*		75,251	73,738	416
8.	65,737	AA*(C)		66,175	66,077	340
1.	7,287	AA*(A)		8,799	7,614	327
2.	16192		AA*	15865	15964	228
2.	27,073		AA*(A)	25,402	26,872	201
9.	101,440		AA*(T)		101,248	192
6.	45,326		AA*	45,207	43,098	119
8.	144,712	AA*(A)		145,939	144,809	97
12.	165,140		AA*	165069	158079	71
11.	148,484	AA*		148,536		52

For AC overhangs (14 Fragments)

*Drd*I#	Location	Overhang	Complement	Nearest *Msp*I	Nearest *Taq*I	Fragment Length
9.	113,009		AC*	109,696	111,008	2,001
6.	100,564		AC*	99,222	99,117	1,342
5.	70,306		AC*	69,207	67,458	1,099
2.	16,508	AC*		17,607	20,496	1,099

FIG. 20

4.	45929	AC*		46933	49057	1004
5.	69,737	AC*		72,665	70,593	856
5.	89,050	AC*		93,107	89,749	699
6.	104,064		AC*	103501	103223	563
2.	17,346		AC*	16,821	14,081	525
2.	26,871		AC*	26,363	21,540	508
8.	80,512		AC*	78,243	80,116	396
10.	113,042	AC*		122,429	113,429	381
5.	51333	AC*		54102	51541	208
4.	61,881		AC*	61,786	60,430	95

For AG overhangs (12 Fragments)

*Drd*I#	Location	Overhang	Complement	Nearest *Msp*I	Nearest *Taq*I	Fragment Length
4.	51345	AG*		57329	59409	5984
7.	55,076		AG*	51,621	53,820	1,256
11.	146,074	AG*		147289	149991	1215
11.	137111		AG*	135970	133640	1141
5.	26,574		AG*	25,682		892
9.	84,646		AG*	DrdI(83,536)	83,821	825
5.	71871	AG*		73210	72675	804
6.	76,750	AG*		77,964	77,104	354
12.	159,685	AG*		160,038	161,212	353
1.	30,506		AG*	30,330	30,080	176
7.	124,720		AG*	124,563	123,299	157
8.	99,628		AG*	99513	99,370	115

For CA overhangs (25 Fragments)

*Drd*I#	Location	Overhang	Complement	Nearest *Msp*I	Nearest *Taq*I	Fragment Length
11.	92,418	CA*(T)		97,628	97,710	5,210
10.	128,190		CA*(G)	111,800	125,432	2,758
8.	92,729		CA*(G)	90,558	90,541	2,171
5.	40,727	CA*(G)		42,854	43,404	2,127
7.	41,602	CA*(T)		50,849	43,487	1,885
11.	114,587	CA*(G)		116,105	116,257	1,518
5.	47,359		CA*(T)	41,626	45,860	1,499
7.	56,328		CA*(A)	52,005	55,150	1,178
12.	174,759		CA*(G)	171,992	173,598	1,161
3.	49,122		CA*(G)		48,199	923
1.	11,050		CA*(T)	10,189	8,861	861

FIG. 20 (cont.)

7.	78,864		CA*(T)		78,112	752
7.	96,506	CA*(A)		98,602	97,059	559
7.	142,867		CA*(G)	135,955	142,371	496
4.	28,263		CA*	27,904	23,795	359
12.	98,198	CA*(A)		98,497	98,862	299
4.	25,393	CA*(G)		25,682		289
8.	83,536	CA*(A)		DrdI(84,646)	83,821	285
7.	104,893	CA*(G)		105,128	105,920	235
5.	40,370		CA*(G)	DrdI(32,405)	40,215	155
6.	60,501		CA*(T)	57,989	60,462	39
7.	68476	CA*		70850	68488	8
3.	27,738	CA*(A)		30,751	27,742	4
6.	77,518	CA*			77522	4
9.	149,703	CA*(A)		151,530	149,707	4

For GA overhangs (15 Fragments)

DrdI#	Location	Overhang	Complement	Nearest *MspI*	Nearest *TaqI*	Fragment Length
6.	25,229	GA*		31,564	30,045	4,816
14.	169,979	GA*		179562	174481	4502
6.	91,681		GA*	88,256	81,884	3,419
5.	97330		GA*	94353	89615	2977
4.	29,833	GA*		41,626	31,251	1,418
4.	66,836		GA*	65,504	62,654	1,332
13.	166,309	GA*		167668	166451	1311

Figure 20 (cont.)

9.	132,110	GA*		133,806	132,976	866
8.	139,856		GA*	139,346	139,218	510
11.	153,548	GA*		153,789	160,722	241
4.	42,388	GA*		42,584	DrdI (42,586)	(196)
9.	102,318		GA*	98,975	102,155	163
10.	107,020		GA*	106,882	105,288	138
10.	138,792		GA*	137757	138715	77
8.	105,928		GA*	105,592	105,920	8

For GG overhangs (12 Fragments)

DrdI#	Location	Overhang	Complement	Nearest *MspI*	Nearest *TaqI*	Fragment Length
3.	33,306	GG*		38,218	40,389	4,918
7.	83,703	GG*		87,372	90,806	3,669
12.	154,063		GG*	142,944	150,402	3,661
2.	19,383		GG*	13,868	17,667	1,710
6.	49,989	GG*		51,421	51,451	1,432
9.	74,360	GG*		75,697	75,962	1,337

FIG. 20 (cont.)

1.	5,385	GG*		6,381	6,249	864
13.	180,666		GG*	179,917	177,380	749
3.	43,961	GG*		48,573	44,652	691
2.	41,448	GG*		42,084	42,010	562
10.	137,833		GG*	137,329	136,062	504
5.	89,627		GG*	80,801	89,331	294

FIG. 20 (cont.)

Determining four unique singlet *DrdI* sequences from two overlapping doublet BAC sequences.

```
Concordant sequences: Doublet to Doublet.

1. TCGTCCTCAGGAACTGAAGCTATATAATCAGTTAAGTCCCTGCTTCTGATCTCTTCTGATTTTCTTCTAAGAAGAGAATA
2. GTGTCAAGTAAAGAAGTACAGCAGATAAGTAAAACGGAAAAAAATAATGAAAGAATTACAAAGGAAGACTAAGGAAAGAG
   ddSSSdsiisdsisdsdssididddisiidsidssssdddsiddSiiddiddiiddSsississdidsddddsdssdSdis
3. AAGTCTACAATCAAGAGGCCAACTGATTCCATGTCTGGTGAGGGTCTATTTCCTGGTGCATAGATGGCTCCTTCTCACTG
2. GTGTCAAGTAAAGAAGTACAGCAGATAAGTAAAACGGAAAAAAATAATGAAAGAATTACAAAGGAAGACTAAGGAAAGAG

From above 2 BACs, sequence #2 is:

         CA   A      C A   A TC  T         G   CT  T  CT   G  T   T             T
2? GTGTCAAGTAAAGAAGTACAGCAGATAAGTAAAACGGAAAAAAATAATGAAAGAATTACAAAGGAAGACTAAGGAAAGAG

Concordant sequences: Doublet to Doublet.

3. AAGTCTACAATCAAGAGGCCAACTGATTCCATGTCTGGTGAGGGTCTATTTCCTGGTGCATAGATGGCTCCTTCTCACTG
2. GTGTCAAGTAAAGAAGTACAGCAGATAAGTAAAACGGAAAAAAATAATGAAAGAATTACAAAGGAAGACTAAGGAAAGAG
   dsSSSdsssSsddsiddisdisdsisisddsisisdsdisSsddsssdsdiddddisdsssSSsiisdiidsddsdsdss
3. AAGTCTACAATCAAGAGGCCAACTGATTCCATGTCTGGTGAGGGTCTATTTCCTGGTGCATAGATGGCTCCTTCTCACTG
4. TAGTCCTCAATTTCACCATGGATTAAATAACAGAACACAGAGTTACTGTGAGACTTGTGGTAGAAAATCTTTAATTCATT

From above 2 BACs, sequence #3 is:

3? AAGTCTACAATCAAGAGGCCAACTGATTCCATGTCTGGTGAGGGTCTATTTCCTGGTGCATAGATGGCTCCTTCTCACTG
              A  A  G   A A    A A    A          A    T         AA  CT
         CA   A      C A   A TC  T         G   CT  T  CT   G  T   T             T
2? GTGTCAAGTAAAGAAGTACAGCAGATAAGTAAAACGGAAAAAAATAATGAAAGAATTACAAAGGAAGACTAAGGAAAGAG

By comparing the consensus sequence between 2 and 3, one can determine the overlap.
In this case, only two positions are indeterminate (A or T).  Hence 2 and 3 are:

                                    T                                       T
2= GTGTCAAGTAAAGAAGTACAGCAGATAAGTAAAACGGAAAAAAATAATGAAAGAATTACAAAGGAAGACTAAGGAAAGAG

                                    A                                       A
3= AAGTCTACAATCAAGAGGCCAACTGATTCCATGTCTGGTGAGGGTCTATTTCCTGGTGCATAGATGGCTCCTTCTCACTG

and by subtraction, one can determine 1 and 4:

                                    A                                       A
1= TCGTCCTCAGGAACTGAAGCTATATAATCAGTTAAGTCCCTGCTTCTGATCTCTTCTGATTTTCTTCTAAGAAGAGAATA

                                    T                                      .T
4= TAGTCCTCAATTTCACCATGGATTAAATAACAGAACACAGAGTTACTGTGAGACTTGTGGTAGAAAATCTTTAATTCATT
```

FIG. 21

Determining three unique singlet *DrdI* sequences from overlapping doublet and triplet BAC sequences.

Concordant sequences: Doublet to Doublet.

```
1. TCGTCCTCAGGAACTGAAGCTATATAATCAGTTAAGTCCCTGCTTCTGATCTCTTCTGATTTTCTTCTAAGAAGAGAATA
2. GTGTCAAGTAAAGAAGTACAGCAGATAAGTAAAACGGAAAAAAATAATGAAAGAATTACAAAGGAAGACTAAGGAAAGAG
   ddSSSdsiisdsisdsdssididddisiidsidssssdddsiddSiiddiddiiddSississdidsddddsdssdSdis
3. AAGTCTACAATCAAGAGGCCAACTGATTCCATGTCTGGTGAGGGTCTATTTCCTGGTGCATAGATGGCTCCTTCTCACTG
2. GTGTCAAGTAAAGAAGTACAGCAGATAAGTAAAACGGAAAAAAATAATGAAAGAATTACAAAGGAAGACTAAGGAAAGAG
```

From above 2 BACs, sequence #2 is:

```
          CA   A      C A   A TC  T         G   CT  T  CT   G  T   T              T
2? GTGTCAAGTAAAGAAGTACAGCAGATAAGTAAAACGGAAAAAAATAATGAAAGAATTACAAAGGAAGACTAAGGAAAGAG
```

Concordant sequences: Doublet to Triplet.

```
3. AAGTCTACAATCAAGAGGCCAACTGATTCCATGTCTGGTGAGGGTCTATTTCCTGGTGCATAGATGGCTCCTTCTCACTG
2. GTGTCAAGTAAAGAAGTACAGCAGATAAGTAAAACGGAAAAAAATAATGAAAGAATTACAAAGGAAGACTAAGGAAAGAG
   iiSSSdssiSdddsiddisdisidisiidisidisdsdissiiisisisdiiddiisdssisssiisiiiddddddsdds
3. AAGTCTACAATCAAGAGGCCAACTGATTCCATGTCTGGTGAGGGTCTATTTCCTGGTGCATAGATGGCTCCTTCTCACTG
4. TAGTCCTCAATTTCACCATGGATTAAATAACAGAACACAGAGTTACTGTGAGACTTGTGGTAGAAAATCTTTAATTCATT
5. GTGTCATCTAGCTATAAATCTAAAGATAATAATAAAATTGGAAAGATTTTCATCAGATAGACTTTTAACACCAAGCTTGA
```

From above 2 BACs, sequence #3 is:

```
3? AAGTCTACAATCAAGAGGCCAACTGATTCCATGTCTGGTGAGGGTCTATTTCCTGGTGCATAGATGGCTCCTTCTCACTG
   GT     T     A  A  G A A AA T A A    A  AAA A T  AA  AT    A   AA ACT
          CA   A      C A   A TC  T         G   CT  T  CT   G  T   T              T
2? GTGTCAAGTAAAGAAGTACAGCAGATAAGTAAAACGGAAAAAAATAATGAAAGAATTACAAAGGAAGACTAAGGAAAGAG
```

By comparing the consensus sequence between 2 and 3, one can determine the overlap. In this case, only two positions are indeterminate (A or T). Hence 2 and 3 are:

```
          A                   T   T         G   C              T   T
2= GTGTCAAGTAAAGAAGTACAGCAGATAAGTAAAACGGAAAAAAATAATGAAAGAATTACAAAGGAAGACTAAGGAAAGAG

          T                   A   A         A   A              A   A
3= AAGTCTACAATCAAGAGGCCAACTGATTCCATGTCTGGTGAGGGTCTATTTCCTGGTGCATAGATGGCTCCTTCTCACTG
```

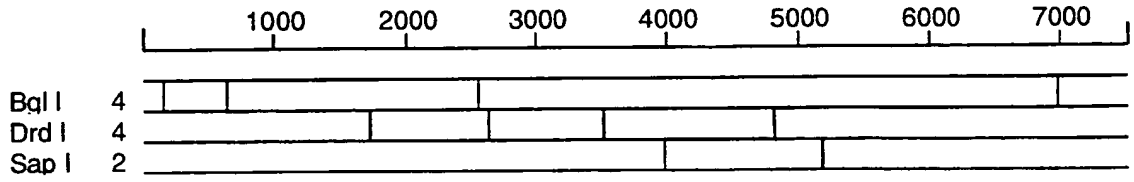
and by subtraction, one can determine 1 is:

```
          T                   A   A         A   A              A   A
1= TCGTCCTCAGGAACTGAAGCTATATAATCAGTTAAGTCCCTGCTTCTGATCTCTTCTGATTTTCTTCTAAGAAGAGAATA
```

From the above data, one cannot determine sequence 4 & 5, although one can reduce it to a doublet sequence by subtracting sequence 3. The alignment of this triplet BAC with another singlet or doublet from the neighboring BAC on the other side (i.e. 5 alone or 5 & 6 doublet) will allow one to decipher sequences 4, 5, and 6

FIG. 22

*Bgl*I, *Drd*I, and *Sap*I sites in the pBeloBAC11 cloning vector.



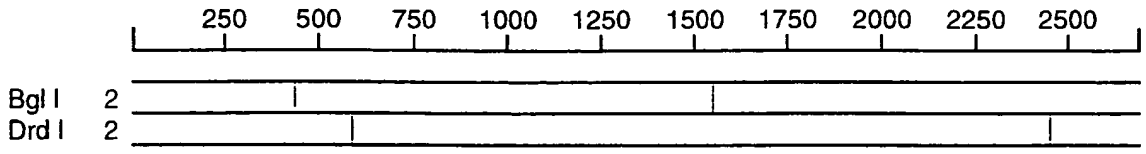
*Bgl*I#	Location	Nearby Site	Overhang (*Bgl*I)	Overlapping Site	Complement (*Bgl*I)	Nearby Site
1.	155	*Fsp*I	TTC		GAA	*Nar*I
2.	634		CCC	*Xma*I	GGG	
3.	2,533		TGT	*Stu*I	ACA	
4.	6,982		TGC	*Ngo*MIV	GCA	

*Drd*I#	Location	Nearby Site	Overhang (*Drd*I)	Overlapping Site	Complement (*Drd*I)	Nearby Site
1.	1,704	*Alw*NI	AA		TT	
2.	2,616		TC		GA	
3.	3,511		GA		TC	*Eco*RI
4.	4,807		TG	*Bsp*HI	CA	

*Sap*I#	Location	Nearby Site	Overhang (*Sap*I)	Overlapping Site	Complement (*Sap*I)	Nearby Site
1.	3,964	*Dra*I	TAT		ATA	
2.	5,174		ACT		AGT	*Bcl*I

FIG. 23

BglI, *DrdI*, and *SapI* sites in the pUC19 cloning vector.



BglI#	Location	Nearby Site	Overhang (*BglI*)	Overlapping Site	Complement (*BglI*)	Nearby Site
1.	429	*Nar*I	GAA		TTC	*Fsp*I
2.	1,547		TTC	*Msp*I	GAA	

DrdI#	Location	Nearby Site	Overhang (*DrdI*)	Overlapping Site	Complement (*DrdI*)	Nearby Site
1.	582		GC		GC	
2.	2,450		GA	*Taq*I	TC	

*Sap*I sites: None

FIG. 24

Sequencing *Bam*HI islands in random BAC clones

1. Cut BAC DNA with *Bam*HI in the presence of linkers and T4 ligase. Linker for *Bam*HI site is not phosphorylated. Biochemical selection assures that most sites contain linkers.

2. Inactivate T4 ligase and *Bam*HI endonuclease at 65°C for 10 min, melt off unligated linker strand. Add *Taq* polymerase and dNTPs and fill in 3' ends. PCR amplify using primers containing ribose U replacing dT, dNTPs, and *Taq* polymerase. Add 0.1N NaOH and heat to 95 °C for 5 min to destroy unused primers.

3. Neutralize and dilute. Anneal sequencing primer

   which extends past the *Bam*HI site linker by two bases and perform a cycle-sequencing reaction. (Separate reactions are performed using primers containing other two base extensions).

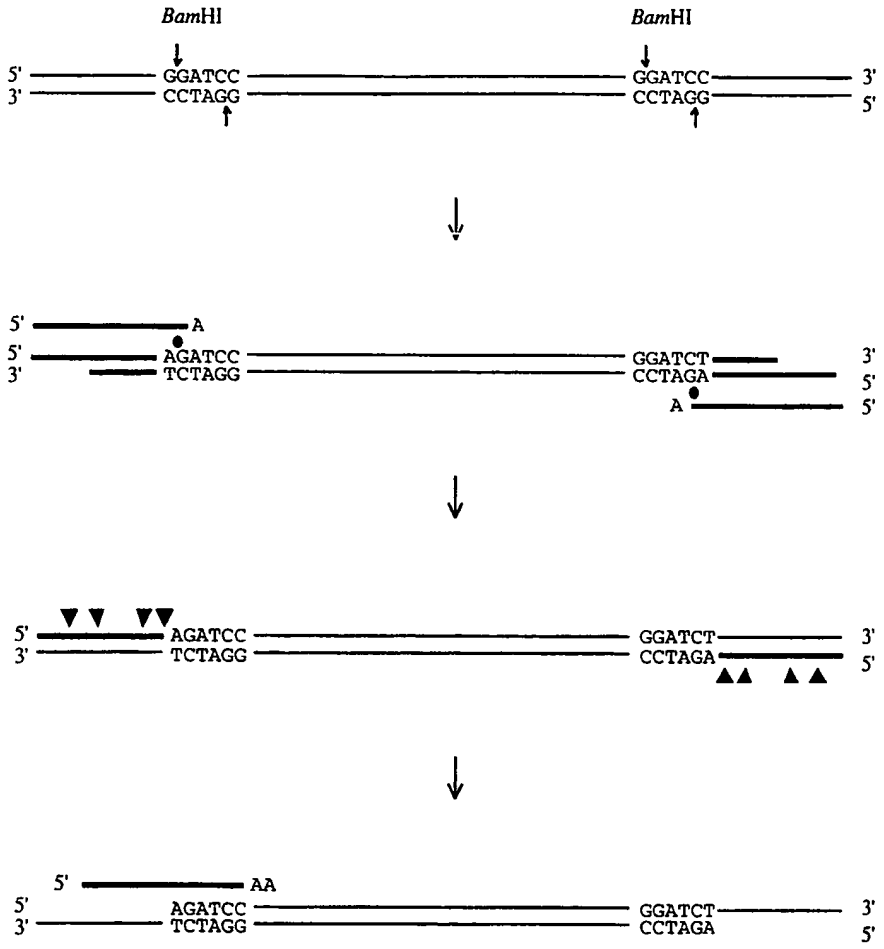
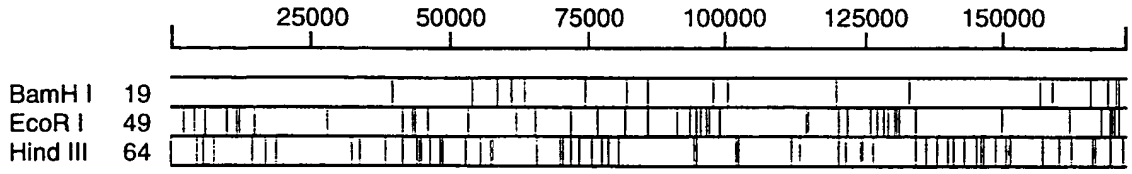


FIG. 25

*Eco*RI, *Hind*III, and *Bam* HI site frequencies in a sequenced BAC from 7q31.

RG253B13, 7q31 Met Oncogene
19 *Bam*HI Sites in 171,905 bp



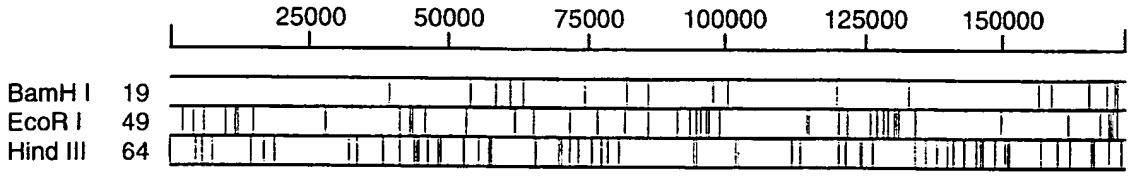

```
Enzyme              Freq       Position(s)

BamH I               19     :    39474     53874     53955     58547
    ↓                       :    61411     63629     74716     82491
  G GATC C                  :    86169     97907    100558    120206
  C CTAG G                  :   132953    156707    159016    165913
        ↑                   :   169171    170414    170908

Number of fragments 4 kb or less:  9
```

*Bam*HI	Location#1	Location#2	+ 2 bases	Complement + 2 bases
1.	53,874	53,955	AT^{x}	TG^{x}
2.	58, 547	61,411	$TA^{@}$	$AA^{@}$
3.	61,411	63,629	TG^{x}	AT^{x}
4.	82,491	86,169	TG^{x}	$CT^{\#}$
5.	97,907	100,558	$AC^{@}$	$TT^{@}$
6.	156,707	159,016	$CA^{@}$	$AG^{@}$
7.	165,913	169,171	TG^{x}	AT^{x}
8.	169,171	170,414	$TC^{\#}$	$TC^{\#}$
9.	170,414	170,908	$CT^{\#}$	TG^{x}

Clusters: (2, 3); (7, 8, 9)

	*Bam*HI
$^{@}$Same + 2 bases next to site within BAC used exactly once (singlet).	6
$^{\#}$Same + 2 bases next to site within BAC used exactly twice (doublet).	2
^{x}Same + 2 bases next to site within BAC used more than twice.	2

FIG. 26



EcoR I	49	:	2446	4350	6140	6158
↓		:	6225	10073	12053	12399
G AATT C		:	15083	28087	41401	43549
C TTAA G		:	43806	46037	53312	62042
↑		:	65700	72180	77101	81978
		:	86301	91655	93891	94983
		:	95739	96841	97167	99214
		:	114696	114949	115133	115232
		:	120578	122208	126085	127496
		:	128732	129314	130523	130710
		:	131286	134360	150100	162281
		:	167783	169521	169653	170292
		:	170998			

Number of fragments 4 kb or less: 34

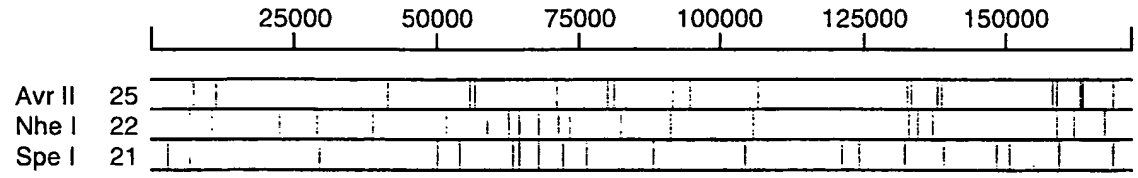
Hind III	64	:	1	321	4834	5918
↓		:	7959	14843	16895	18994
A AGCT T		:	32159	33703	38308	41512
T TCGA A		:	44158	44521	44717	46402
↑		:	48209	48692	52752	55612
		:	57379	57727	65779	70218
		:	70601	71947	73380	75933
		:	77773	78860	80726	94474
		:	94886	102267	102578	112246
		:	113833	120486	121556	121647
		:	124186	124409	124818	126795
		:	134126	136011	137970	140077
		:	141184	143075	145328	146005
		:	146673	148906	150711	150993
		:	151617	157093	160311	162518
		:	166369	166672	169514	171900

Number of fragments 4 kb or less: 52

FIG. 26 (cont.)

*Avr*II, *Nhe*I, and *Spe*I site frequencies in a sequenced BAC from 7q31.

RG253B13, 7q31 Met Oncogene
25 *Avr*II, 22 *Nhe*I, and 21 *Spe*I Sites in 171,905 bp



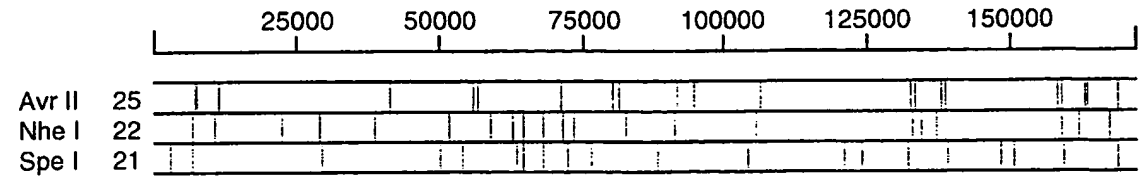

```
Enzyme          Freq      Position(s)

Avr II           25   :     7350      7990     11781     41276
   ↓                  :    56073     56739     71378     80285
  C CTAG G            :    80378     80418     81455     92044
  G GATC C            :    95088    106812    132860    133491
       ↑              :   138089    138866    138891    138919
                      :   158473    159109    163153    163762
                      :   168991

Number of fragments 4 kb or less:  14  (Clustering)
```

*Avr*II	Location#1	Location#2	+ 2 bases	Complement + 2 bases
1.	7,350	7,990	CT^{x}	AA^{x}
2.	7,990	11,781	CC$^{@}$	CT^{x}
3.	56,073	56,739	CA$^{\#}$	TG$^{@}$
4.	80,285	80,378	TT^{x}	AC$^{\#}$
5.	80,378	80,418	CA	CA (40 bp fragment)
6.	80,418	81,455	AC$^{\#}$	AA^{x}
7.	92,044	95,088	GG$^{@}$	TC$^{@}$
8.	132,860	133,491	TT^{x}	AA^{x}
9.	138, 089	138, 866	CT^{x}	TT^{x}
10.	138, 866	138,891	TG	TG (25 bp fragment)
11.	138, 891	138,919	CT	AG (28 bp fragment)
12.	158, 473	159, 109	AA^{x}	TT^{x}
13.	159, 109	163,153	CA$^{\#}$	TA$^{@}$
14.	163,153	163,762	AA^{x}	TT^{x}

Clusters: (4, 5, 6); (9, 10, 11); (13, 14)

	*Avr*II
$^{@}$Same + 2 bases next to site within BAC used exactly once (singlet).	5
$^{\#}$Same + 2 bases next to site within BAC used exactly twice (doublet).	2
^{x}Same + 2 bases next to site within BAC used more than twice.	3

FIG. 27



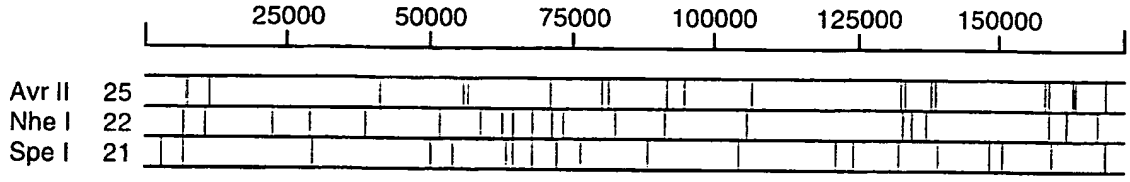

```
Nhe I            22      :     7114     10879     22730     29080
   ↓                     :    38661     51766     58900     62751
  G CTAG C               :    64798     68351     71494     73609
  C GATC G               :    82697     91479    106192    132980
       ↑                 :   134667    134793    137390    158989
                         :   161975    167497

Number of fragments 4 kb or less:  10  (Clustering)
```

*Nhe*I	Location#1	Location#2	+ 2 bases	Complement + 2 bases
1.	7,114	10,879	TT#	TC^{X}
2.	58,900	62,751	TG#	CA^{X}
3.	62,751	64,798	AC^{X}	AC^{X}
4.	64,798	68,351	TC^{X}	TC^{X}
5.	68,351	71,494	AC^{X}	TG#
6.	71,494	73,609	TA@	CA^{X}
7.	132,980	134,667	CA^{X}	AA@
8.	134,667	134,793	GG@	AG#
9.	134,793	137,390	TT#	AC^{X}
10.	158,989	161,975	CA^{X}	AG#

Clusters: (3, 4, 5, 6); (7, 8, 9)

	*Nhe*I
@Same + 2 bases next to site within BAC used exactly once (singlet).	3
#Same + 2 bases next to site within BAC used exactly twice (doublet).	3
^{X}Same + 2 bases next to site within BAC used more than twice.	3

FIG. 27 (cont.)




```
Spe I          21    :    3173     7256     29438     50198
  ↓                  :   54057    63422     64771     68328
  A CTAG T           :   72447    76712     88296    104546
  T GATC A           :  121378   124275    132360    139059
        ↑            :  139107   148566    150563    159612
                     :  169084

Number of fragments 4 kb or less:  9    (Clustering)
```

*Spe*I	Location#1	Location#2	+ 2 bases	Complement + 2 bases
1.	3,173	7,256	TC#	GA^{x}
2.	50,198	54,057	TG#	GG^{x}
3.	63,422	64,777	GA^{x}	GG^{x}
4.	64,777	68,328	CA@	GG^{x}
5.	68,328	72,447	TT^{x}	TT^{x}
6.	72,447	76,712	GT@	GC@
7.	121,378	124,275	GA^{x}	TC#
8.	139,059	139,107	AT	AC (48 bp fragment)
9.	148,566	150,563	TG#	TT^{x}

Clusters: (3, 4, 5, 6)

	*Spe*I
@Same + 2 bases next to site within BAC used exactly once (singlet).	3
#Same + 2 bases next to site within BAC used exactly twice (doublet).	3
^{x}Same + 2 bases next to site within BAC used more than twice.	3

FIG. 27 (cont.)

Sequencing *Bsi*HKAI islands in random BAC clones

1. Cut BAC DNA with *Bsi*HKAI in the presence of linkers and T4 ligase. Linker for *Bsi*HKAI site is phosphorylated and contains a 3' AGCA overhang. Biochemical selection assures that most sites contain linkers.

2. Inactivate T4 ligase and *Bsi*HKAI endonuclease at 95°C for 5 min. PCR amplify using primers containing ribose U replacing dT, dNTPs, and *Taq* polymerase. Add 0.1N NaOH and heat to 95 °C for 5 min to destroy unused primers.

3. Neutralize and dilute. Anneal sequencing primer which extends past the *Bsi*HKAI site linker by two bases and perform a cycle-sequencing reaction. (Separate reactions are performed using primers containing other two base extensions).

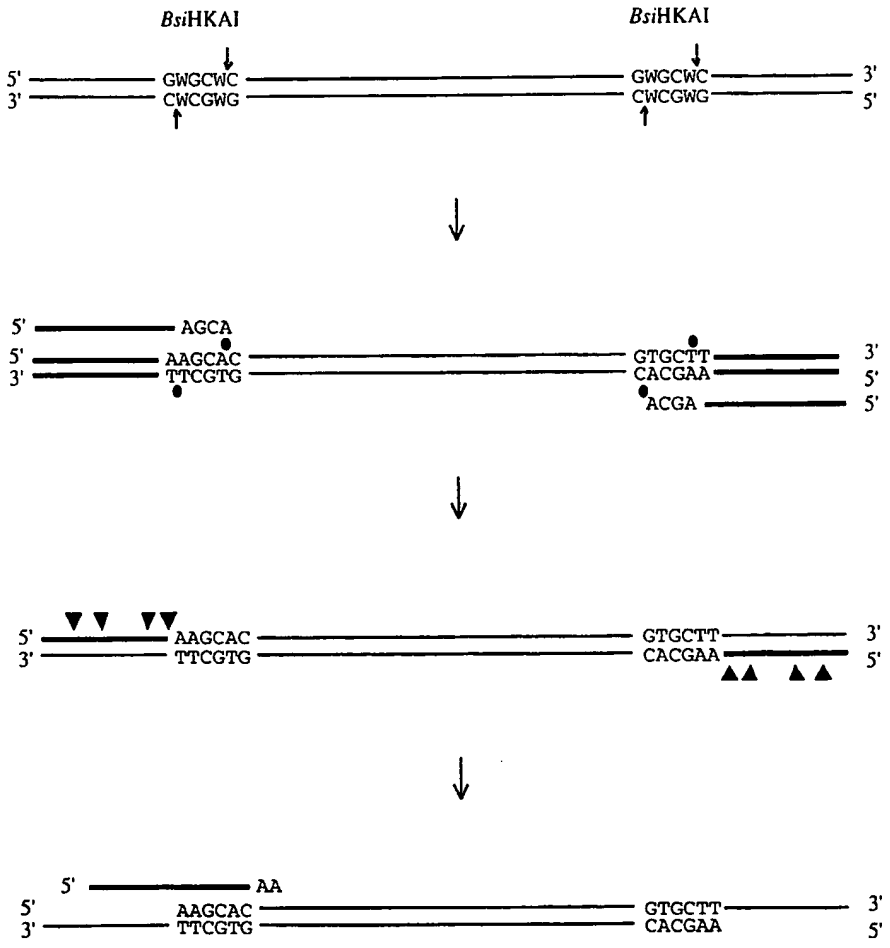
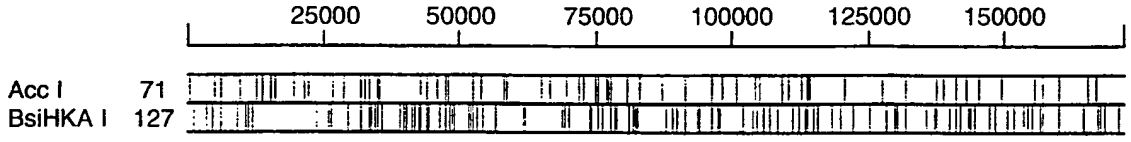


FIG. 28

*Acc*I and *Bsi*HKAI site frequencies in a sequenced BAC from 7q31.

RG253B13, 7q31 Met Oncogene
71 *Acc*I and 127 *Bsi*HKAI Sites in 171,905 bp

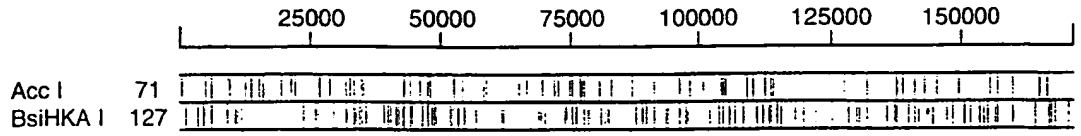


Enzyme	Freq		Position(s)			
Acc I	71	:	523	5182	6465	9711
↓		:	12950	13976	15332	16332
GT MK AC		:	19814	21540	22269	22322
CA KM TG		:	26959	28705	32048	32661
↑		:	33298	33310	34799	35425
		:	42895	44110	46004	47636
		:	47861	52446	54000	58216
		:	58826	65238	66475	69750
		:	71833	72783	74938	75538
		:	77087	77368	77642	80744
		:	82917	87470	91592	96498
		:	98545	100882	100965	104551
		:	104725	105186	109580	110415
		:	112720	114135	114242	120913
		:	127597	131831	137724	139036
		:	141043	142923	142963	145284
		:	149681	155647	157032	160140
		:	165449	167062	167292	

*Acc*I#	Location#1	Location#2	AG#1+ 2	AG#2 + 2
1.	13,976	15,332	TT#	AT#
2.	33,298	33,310	(10 bp fragment)	
3.	35,425	42,895	(Too long)	
4.	69,750	71,833	TT#	AA@
5.	96,498	98,545	CC@	AT@
6.	109,580	110,415	AT#	TG@

	*Acc*I
@Same + 2 bases next to site within BAC used exactly once (singlet).	4
#Same + 2 bases next to site within BAC used exactly twice (doublet).	2
xSame + 2 bases next to site within BAC used more than twice.	0

FIG. 29



Enzyme	Freq	Position(s)			
BsiHKA I ↓ G WGCW C C WCGW G ↑	127	: 1200	1274	3588	4610
		: 6151	9251	9358	10891
		: 11182	12046	23820	26072
		: 26538	29548	31865	33417
		: 33620	33828	34406	34818
		: 35750	39076	39888	40291
		: 41356	41605	41622	41723
		: 42439	43101	43155	43959
		: 44003	44572	46346	47692
		: 48495	48608	49119	51943
		: 52138	52540	53172	53348
		: 54384	56608	61639	61987
		: 68891	69195	70155	73864
		: 74122	75448	76167	77810
		: 78326	78825	81275	81950
		: 82251	82594	87958	89375
		: 90017	91434	91584	93846
		: 94001	96276	97766	97942
		: 102220	104114	105012	106105
		: 107321	108501	111466	112396
		: 113542	114132	115157	116106
		: 118786	120094	122269	122357
		: 122376	122400	125590	128460
		: 130102	130144	130366	131806
		: 135930	137267	137611	139881
		: 141326	141747	143572	143995
		: 144453	144701	147329	148398
		: 150702	150741	151888	153643
		: 154630	155122	156946	157058
		: 160171	160400	164987	167605
		: 167618	167660	167683	168011
		: 168643	168776	171471	

*Bsi*HKAI	Location#1	Location#2	AGCA#1+ 2	AGCA#2 + 2
1.	3,588	4,610	AC#	TT#
2.	23,820	26,072	AA@	TG#
3.	43,959	44,003	TT	AA (44 bp fragment)
4.	48,608	49,119	AG@	GA@
5.	52,138	52,540	CT@	GG@
6.	76,167	77,810	AC#	TT#
7.	102,220	104,114	CC	CC (24 bp fragment)
8.	155,122	156,946	AT@	TG#

	*Bsi*HKAI
@Same + 2 bases next to site within BAC used exactly once (singlet).	6
#Same + 2 bases next to site within BAC used exactly twice (doublet).	3
xSame + 2 bases next to site within BAC used more than twice.	0

FIG. 29 (cont.)

Sequencing *San*DI islands in random BAC clones

1. Cut BAC DNA with *Msp*I and *San*DI in the presence of linkers and T4 ligase. Linker for *San*DI site is phosphorylated and contains a 5' GTC overhang. Linker for *Msp*I site is not phosphorylated, and contains a bubble. Biochemical selection assures that most sites contain linkers.

2. Inactivate T4 ligase and restriction endonucleases at 95°C for 5 min. PCR amplify using primers containing ribose U replacing dT, dNTPs, and *Taq* polymerase. Add 0.1N NaOH and heat to 95 °C for 5 min to destroy unused primers.

3. Neutralize and dilute. Anneal sequencing primer

   which extends past the *San*DI site linker by two bases and perform a cycle-sequencing reaction. (Separate reactions are performed using primers containing other two base extensions).

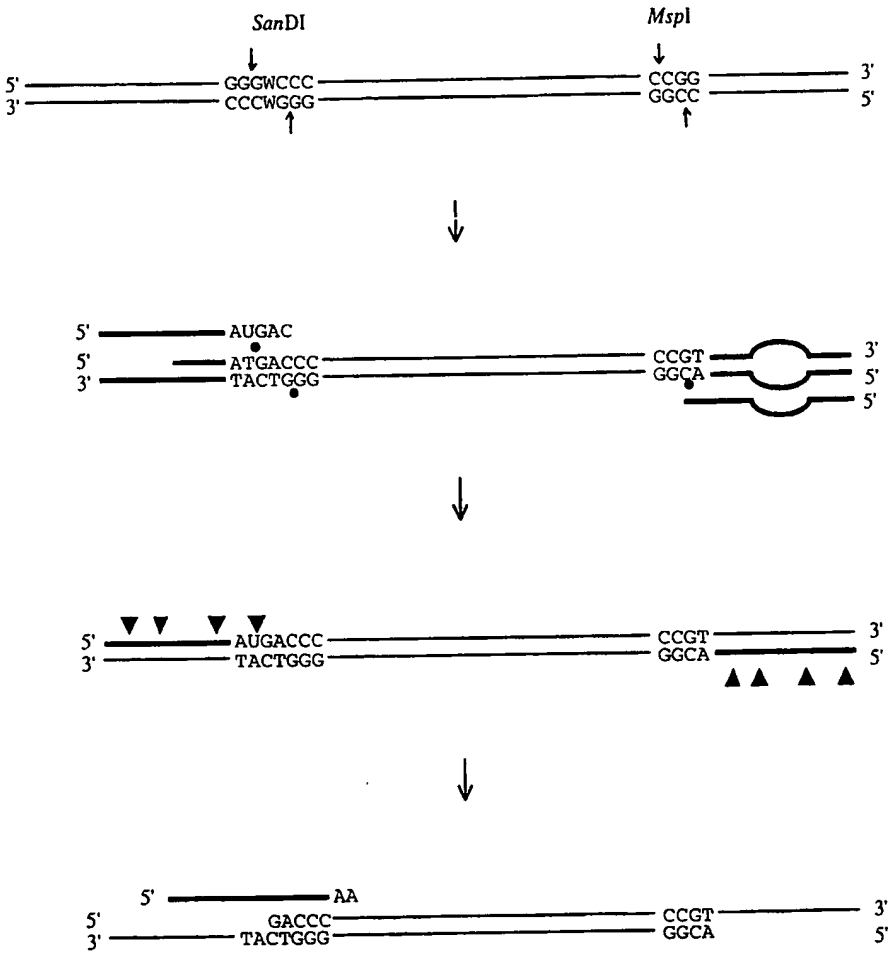
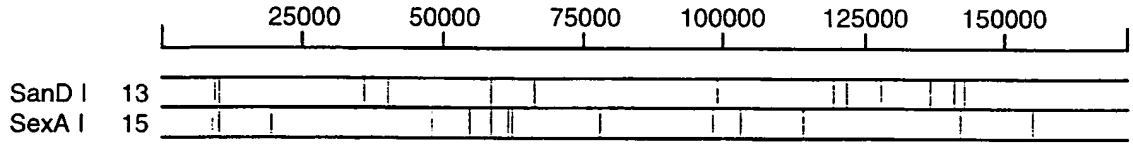


FIG. 30

*San*DI and *Sex*AI site frequencies in a sequenced BAC from 7q31.

RG253B13, 7q31 Met Oncogene
13 *San*DI and 15 *Sex*AI Sites in 171,905 bp

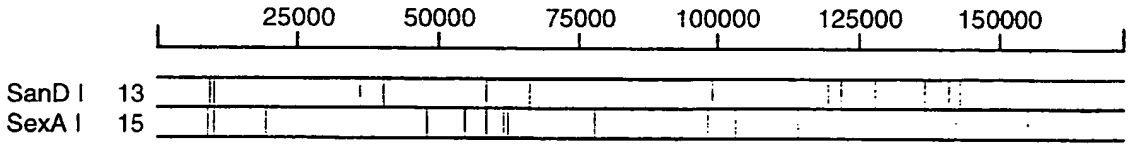


Enzyme	Freq		Position(s)			
SanD I	13	:	9761	10644	36269	40440
↓		:	58583	66380	99267	119927
GG GWC CC		:	122060	128057	137082	140964
CC CWG GG		:	143225			
↑		:				

*San*DI#	Location	GAC + 2 bases
1.	9,761	CT#
2.	10,644	TC#
3.	36,269	AC#
4.	40,440	TC#
5.	58,583	TG#
6.	66,380	CA@
7.	99,267	TG#
8.	119,927	AT@
9.	122,060	CG@
10.	128,057	TA#
11.	137,082	AC#
12.	140,964	CT#
13.	143,225	TA#

	*San*DI
@Same + 2 bases next to site within BAC used exactly once (singlet).	3
#Same + 2 bases next to site within BAC used exactly twice (doublet).	5
xSame + 2 bases next to site within BAC used more than twice.	0

FIG. 31



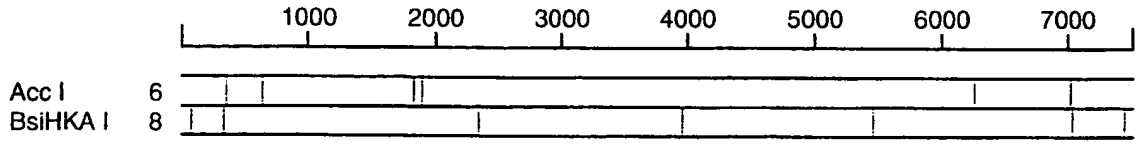
Enzyme	Freq		Position(s)			
SexA I	15	:	9499	10411	19691	47816
↓		:	54773	58714	61533	62534
A CCWGG T		:	78279	98356	103356	114268
T GGWCC A		:	114440	142141	155393	
↑		:				

*Sex*AI#	Location	CCAGG + 2 bases
1.	9,499	TG[@]
2.	10,411	CTX
3.	19,691	TT#
4.	47,816	CC[@]
5.	54,773	CTX
6.	58,714	GG[@]
7.	61,533	GC[@]
8.	62,534	TC[@]
9.	78,279	CT[x]
10.	98,356	TT[#]
11.	103,356	AT[@]
12.	114,268	AA[@]
13.	114,440	GA[#]
14.	142,141	CA[@]
15.	155,393	GA[#]

	*Sex*AI
[@]Same + 2 bases next to site within BAC used exactly once (singlet).	8
[#]Same + 2 bases next to site within BAC used exactly twice (doublet).	2
[x]Same + 2 bases next to site within BAC used more than twice.	1

FIG. 31 (cont.)

*Acc*I and *Bsi*HKAI sites in the pBeloBAC11 cloning vector.




```
Enzyme              Freq        Position(s)
Acc I                 6     :     367      647     1832     1891     6262     7031
    ↓                       :
  GT MK AC                  :
  CA KM TG                  :
       ↑                    :
```

*Acc*I# Location#1 Location#2 AG#1+2 AG#2+2

None with head to head AG overhangs.

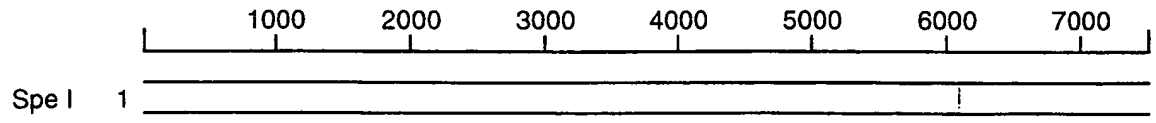
```
BsiHKA I              8     :      91      343     2352     3966     5458     7040
       ↓                    :    7048     7458
  G WGCW C                  :
  C WCGW G                  :
   ↑                        :
```

*Bsi*HKAILocation#1 Location#2 AGCA#1+2 AGCA#2+2

None with head to head AGCA overhangs.

FIG. 32

*Avr*II, *Bam* HI, *Nhe*I, and *Spe*I, sites in the pBeloBAC11 cloning vector.



*Avr*II Location#1 Location#2 + 2 bases Complement + 2 bases

```
Non-cutting enzymes :
 Avr II     Nhe I
```

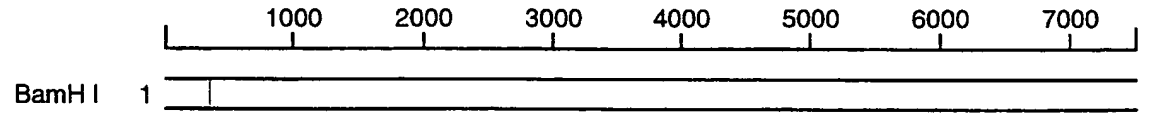
*Nhe*I Location#1 Location#2 + 2 bases Complement + 2 bases

```
Non-cutting enzymes :
 Avr II     Nhe I
```

```
Enzyme            Freq        Position(s)
Spe I               1     :    6090
    ↓                       :
   A CTAG T                 :
   T GATC A                 :
        ↑                   :
```

*Spe*I Location#1 Location#2 + 2 bases Complement + 2 bases

No PCR vector fragment under 4 kb.



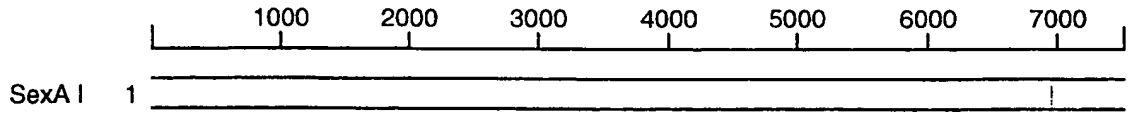

```
Enzyme            Freq        Position(s)
BamH I              1     :    354
    ↓                       :
   G GATC C                 :
   C CTAG G                 :
        ↑                   :
```

*Bam*HI Location#1 Location#2 + 2 bases Complement + 2 bases

No PCR vector fragment under 4 kb.

FIG. 33

*San*DI and *Sex*AI sites in the pBeloBAC11 cloning vector.



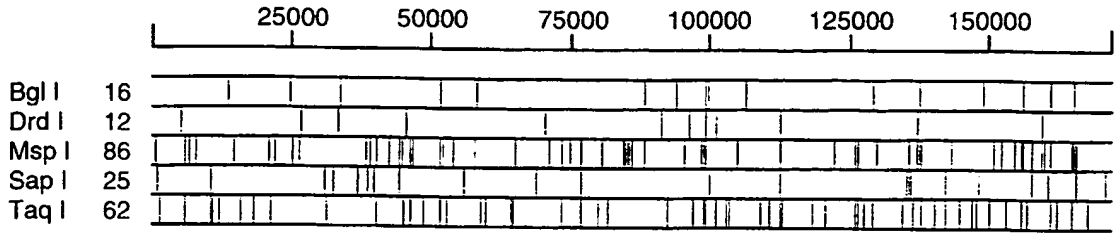
*San*DI#Location A + 2 bases

```
Non-cutting enzymes :
 SanD I
```

```
Enzyme          Freq      Position(s)
SexA I            1    :   6968
    ↓                  :
   A CCWGG T           :
   T GGWCC A           :
         ↑             :
```

*Sex*AI#Location CCAGG + 2 bases
1. 6,968 AT

FIG. 34



Sequence : BAC RG253B13.seq (1 > 171905) 201 Cut Sites

822	Msp I	45534	Drd I	112720	Taq I		
1205	Sap I	46540	Taq I	112849	Sap I		
				112925	Msp I		
1455	Sap I	51614	Msp I				
1744	Taq I	51629	Bgl I	112992	Taq I		
5385	Drd I	51633	Msp I	113048	Drd I		
6249	Taq I			113429	Taq I		
		54087	Msp I				
10904	Taq I	56037	Sap I	127710	Taq I		
10942	Sap I	57978	Msp I	129198	Bgl I		
		58314	Bgl I	129286	Taq I		
10962	Sap I	58886	Taq I				
				134499	Taq I		
11040	Sap I	64973	Msp I	135480	Sap I		
11123	Taq I	68849	Sap I	135615	Sap I		
		70528	Drd I	135890	Msp I		
12529	Taq I	71393	Msp I	136246	Sap I		
13839	Bgl I			136580	Taq I		
14933	Msp I	76855	Taq I	137177	Drd I		
		76929	Sap I	137473	Msp I		
22165	Msp I	77041	Msp I				
25121	Bgl I			137728	Msp I		
25228	Msp I	88256	Msp I	137753	Bgl I		
		88322	Bgl I	137987	Msp I		
26363	Msp I	91681	Drd I				
26871	Drd I	92596	Taq I	140465	Taq I		
31038	Sap I	94140	Bgl I	142242	Sap I		
31368	Taq I	95752	Msp I	142402	Taq I		
		96506	Drd I				
31440	Taq I	97059	Taq I	147961	Taq I		
32606	Sap I			148482	Sap I		
33306	Drd I	99370	Taq I	149252	Bgl I		
33896	Bgl I	99469	Bgl I	150256	Taq I		
37052	Sap I	99513	Msp I				
38218	Msp I	99628	Drd I	156469	Msp I		
38938	Sap I	100051	Bgl I	156583	Bgl I		
39316	Msp I	100257	Sap I	157032	Taq I		
39876	Sap I	101248	Taq I	157977	Sap I		
40280	Msp I	101440	Drd I	158054	Msp I		
		103234	Taq I	159685	Drd I		
42440	Msp I			160038	Msp I		
44332	Sap I	105244	Msp I				
44477	Msp I	106619	Bgl I	160434	Msp I		
		109494	Taq I	160832	Sap I		
45343	Taq I			161212	Taq I		

FIG. 35

161237 Msp I
161467 Bgl I
162462 Taq I

165127 Taq I
165703 Bgl I
165714 Msp I
166152 Sap I
166163 Msp I

168336 Taq I

171459 Sap I

FIG. 35 (cont.)

***Drd*I site:** For AA, AC, AG, CA, GA, and GG overhangs

*Drd*I#	Location	Overhang	Complement	Nearest *Msp*I or *Taq*I	Fragment Length
1.	5,379	GG*	CC	6,249	864
2.	26,865	GT	AC*	26,363	502
3.	33,300	GG*	CC	38,218	4,918#
4.	45,528	AT	AT		
5.	70,522	AT	AT		
6.	91,675	TC	GA*	88,256	3,419
7.	96,500	CA*	TG	97,059	559
8.	99,622	CT	AG*	99,513	115
9.	101,434	TT	AA*	101,248	192
10.	113,042	AC*	GT	113,429	381
11.	137,171	TT	AA*	136,580	597
12.	159,679	AG*	CT	160,038	353

* To obtain sequence information on AA, AC, AG, CA, GA, or GG overhangs in the sense direction, the *Drd*I island is amplified using a downstream *Msp*I or *Taq*I site. For such two base sequences on the complementary strand, the *Drd*I island is amplified using an upstream *Msp*I or *Taq*I site.

Same last 2 bases of 3' overhang within BAC used exactly once (singlet).	3
Same last 2 bases of 3' overhang within BAC used exactly twice (doublet).	3
Same last 2 bases of 3' overhang within BAC used more than twice.	0

***Drd*I site:** For TT, GT, CT, TG, TC, and CC overhangs

*Drd*I#	Location	Overhang	Complement	Nearest *Msp*I or *Taq*I	Fragment Length
1.	5,379	GG	CC*	1,744	3,635
2.	26,865	GT*	AC	31,368	4,503#
3.	33,300	GG	CC*	31,440	1,860
4.	45,528	AT	AT		
5.	70,522	AT	AT		
6.	91,675	TC*	GA	92,596	921
7.	96,500	CA	TG*	95,752	748
8.	99,622	CT*	AG	101,248	1,626
9.	101,434	TT*	AA	103,234	1,800
10.	113,042	AC	GT*	112,992	50#
11.	137,171	TT*	AA	137,473	302
12.	159,679	AG	CT*	158,054	1,625

* To obtain sequence information on TT, GT, CT, TG, TC, or CC overhangs in the sense direction, the *Drd*I island is amplified using a downstream *Msp*I or *Taq*I site. For such two base sequences on the complementary strand, the *Drd*I island is amplified using an upstream *Msp*I or *Taq*I site.

Same last 2 bases of 3' overhang within BAC used exactly once (singlet).	2
Same last 2 bases of 3' overhang within BAC used exactly twice (doublet).	3
Same last 2 bases of 3' overhang within BAC used more than twice.	0

Fragment too small to give interpretable sequence (>80), or too large to amplify properly.

FIG. 35 (cont.)

***BglI* site:** For AAN, CAN, GAN, TAN, AGN, CGN, GGN, and TGN overhangs

BglI#	Location	Overhang	Complement	Nearest *MspI* or *TaqI*	Fragment Length
1.	13,833	TGT*	ACA	14,933	1,100
2.	25,115	ACA	TGT*	22,165	2,950
3.	33,890	GAA*	TTC	37,052	3,162
4.	51,623	TGT*	ACA	51,633	10#
5.	58,308	CTA	TAG*	57,978	330
6.	88,316	TTA	TAA*	88,256	60#
7.	94,134	GGG*	CCC	95,752	1,618
8.	99,463	ACA	TGT*	99,370	93
9.	100,045	ACC	GGT*	99,628	417
10.	106,613	CCA	TGG*	105,244	1,369
11.	129,192	TGT*	ACA	129,286	94
12.	137,747	TCT	AGA*	137,728	19#
13.	149,246	TGT*	ACA	150,256	110
14.	156,577	TTT	AAA*	156,469	108
15.	161,461	CGA*	TCG	162,462	101
16.	165,697	CTG	CAG*	165,127	570

* To obtain sequence information on AAN, CAN, GAN, TAN, AGN, CGN, GGN, or TGN overhangs in the sense direction, the *BglI* island is amplified using a downstream *MspI* or *TaqI* site. For such three base sequences on the complementary strand, the *BglI* island is amplified using an upstream *MspI* or *TaqI* site.

Same last 2 bases of 3' overhang within BAC used exactly once (singlet).	**5**
Same last 2 bases of 3' overhang within BAC used exactly twice (doublet).	**2**
Same last 2 bases of 3' overhang within BAC used more than twice.	1

***BglI* site:** For ACN, CCN, GCN, TCN, ATN, CTN, GTN, and TTN overhangs

BglI#	Location	Overhang	Complement	Nearest *MspI* or *TaqI*	Fragment Length
1.	13,833	TGT	ACA*	12,529	1,304
2.	25,115	ACA*	TGT	25,228	113
3.	33,890	GAA	TTC*	33,306	584
4.	51,623	TGT	ACA*	51,614	9#
5.	58,308	CTA*	TAG	58,886	578
6.	88,316	TTA*	TAA	91,681	3,365
7.	94,134	GGG	CCC*	92,596	1,538
8.	99,463	ACA*	TGT	99,513	50#
9.	100,045	ACC*	GGT	100,257	212
10.	106,613	CCA*	TGG	109,494	2,881
11.	129,192	TGT	ACA*	127,710	1,482
12.	137,747	TCT*	AGA	137,987	240
13.	149,246	TGT	ACA*	148,482	764
14.	156,577	TTT*	AAA	157,032	455
15.	161,461	CGA	TCG*	161,237	224
16.	165,697	CTG*	CAG	165,714	17#

FIG. 35 (cont.)

* To obtain sequence information on ACN, CCN, GCN, TCN, ATN, CTN, GTN, or TTN overhangs in the sense direction, the *BglI* island is amplified using a downstream *MspI* or *TaqI* site. For such three base sequences on the complementary strand, the *BglI* island is amplified using an upstream *MspI* or *TaqI* site.
Same last 2 bases of 3' overhang within BAC used exactly once (singlet). **0**
Same last 2 bases of 3' overhang within BAC used exactly twice (doublet). **3**
Same last 2 bases of 3' overhang within BAC used more than twice. 2
Fragment too small to give interpretable sequence (>80), or too large to amplify properly.

Or, alternatively, mix and match the above to include trinucleotides where the middle base of the upper strand is either A or C, corresponding to the 3' end of the PCR primer.

***BglI* site:** For AAN, CAN, GAN, TAN, ACN, CCN, GCN, and TCN overhangs

BglI#	Location	Overhang	Complement	Nearest *MspI* or *TaqI*	Fragment Length
1.	13,833	**TGT**	**ACA***	12,529	1,304
2.	25,115	**ACA***	**TGT**	25,228	113
3.	33,890	**GAA***	**TTC**	37,052	3,162
4.	51,623	**TGT**	**ACA***	51,614	9#
5.	58,308	**CTA**	**TAG***	57,978	330
6.	88,316	**TTA**	**TAA***	88,256	60#
7.	94,134	**GGG**	**CCC***	92,596	1,538
8.	99,463	**ACA***	**TGT**	99,513	50#
9.	100,045	**ACC***	**GGT**	100,257	212
10.	106,613	**CCA***	**TGG**	109,494	2,881
11.	129,192	**TGT**	**ACA***	127,710	1,482
12.	137,747	**TCT***	**AGA**	137,987	240
13.	149,246	**TGT**	**ACA***	148,482	764
14.	156,577	**TTT**	**AAA***	156,469	108
15.	161,461	**CGA**	**TCG***	161,237	224
16.	165,697	**CTG**	**CAG***	165,127	570

* To obtain sequence information on AAN, CAN, GAN, TAN, ACN, CCN, GCN, or TCN overhangs in the sense direction, the *BglI* island is amplified using a downstream *MspI* or *TaqI* site. For such three base sequences on the complementary strand, the *BglI* island is amplified using an upstream *MspI* or *TaqI* site.
Same last 2 bases of 3' overhang within BAC used exactly once (singlet). **3**
Same last 2 bases of 3' overhang within BAC used exactly twice (doublet). **3**
Same last 2 bases of 3' overhang within BAC used more than twice. 1
Fragment too small to give interpretable sequence (>80), or too large to amplify properly.

FIG. 35 (cont.)

For AA, AC, AG, AT, GA, GC, GG and GT overhangs

*Sap*I#	Location	*Sap*I Overhang	Ligated Complement	Nearest *Msp*I or *Taq*I	Fragment Length
1.	1,198	CTA	**TAG*** down	No	
2.	1,456	AGG	**CCT** up	No	
3.	10,943	GCT	**AGC*** up	10,904	39#
4.	10,955	GCT	**ACG** down	No	
5.	11,041	CAA	**TTG** up	No	
6.	31,031	AAT	**ATT** down	31,368	
7.	32,599	GAT	**ATC** down	No	
8.	37,053	AGA	**TCT** up	No	
9.	38,931	GGG	**CCC** down	39,316	
10.	39,877	ATC	**GAT*** up	39,316	571
11.	44,325	CTT	**AAG*** down	44,477	152
12.	56,040	ACA	**TGT*** down	57,978	1,938
13.	68,850	ACC	**GGT*** up	64,973	3,877
14.	76,930	GTG	**CAC*** up	76,855	75#
15.	100,250	GGG	**CCC** down	101,248	
16.	112,850	GAT	**ATC** up	112,720	
17.	135,473	ACA	**TGT*** down	No	
18.	135,608	GGA	**TCC** down	135,890	
19.	136,239	TTG	**CAA*** up	135,890	349
20.	142,243	GCC	**GGC*** up	140,465	1,778
21.	148,475	GCG	**CGC*** down	150,256	1,781
22.	157,978	TCT	**AGA*** up	157,032	946
23.	160,833	ACC	**GGT*** up	160,434	399
24.	166,153	ATT	**AAT*** up	165,714	439
25.	171,460	GTT	**AAC*** up	168,336	3,124

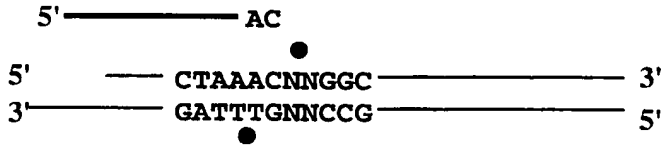
* To obtain sequence information on AA, AC, AG, AT, GA, GC, GG or GT overhangs in the sense direction, the *Sap*I island is amplified using a downstream *Msp*I or *Taq*I site. For such two base sequences on the complementary strand, the *Bgl*I island is amplified using an upstream *Msp*I or *Taq*I site.

Same last 2 bases of 3' overhang within BAC used exactly once(singlet). **3**
Same last 2 bases of 3' overhang within BAC used exactly twice (doublet). **3**
Same last 2 bases of 3' overhang within BAC used more than twice. 1

Fragment too small to give interpretable sequence (>80), or too large to amplify properly.

FIG. 35 (cont.)

Three degrees of specificity in amplifying a *BglI* representation.



1. Ligation of the top strand requires perfect complementarity at the penultimate base to the 3' side of the junction (20-fold specificity).
2. Ligation of the bottom strand requires perfect complementarity at the 3' side of the junction (50-fold specificity).
3. Extension of polymerase off the sequencing primer is most efficient if the 3' base is perfectly matched (10 to 100-fold specificity).

FIG. 36

Scheme 1 for sequencing *Drd*I and *Bgl*I generated representations

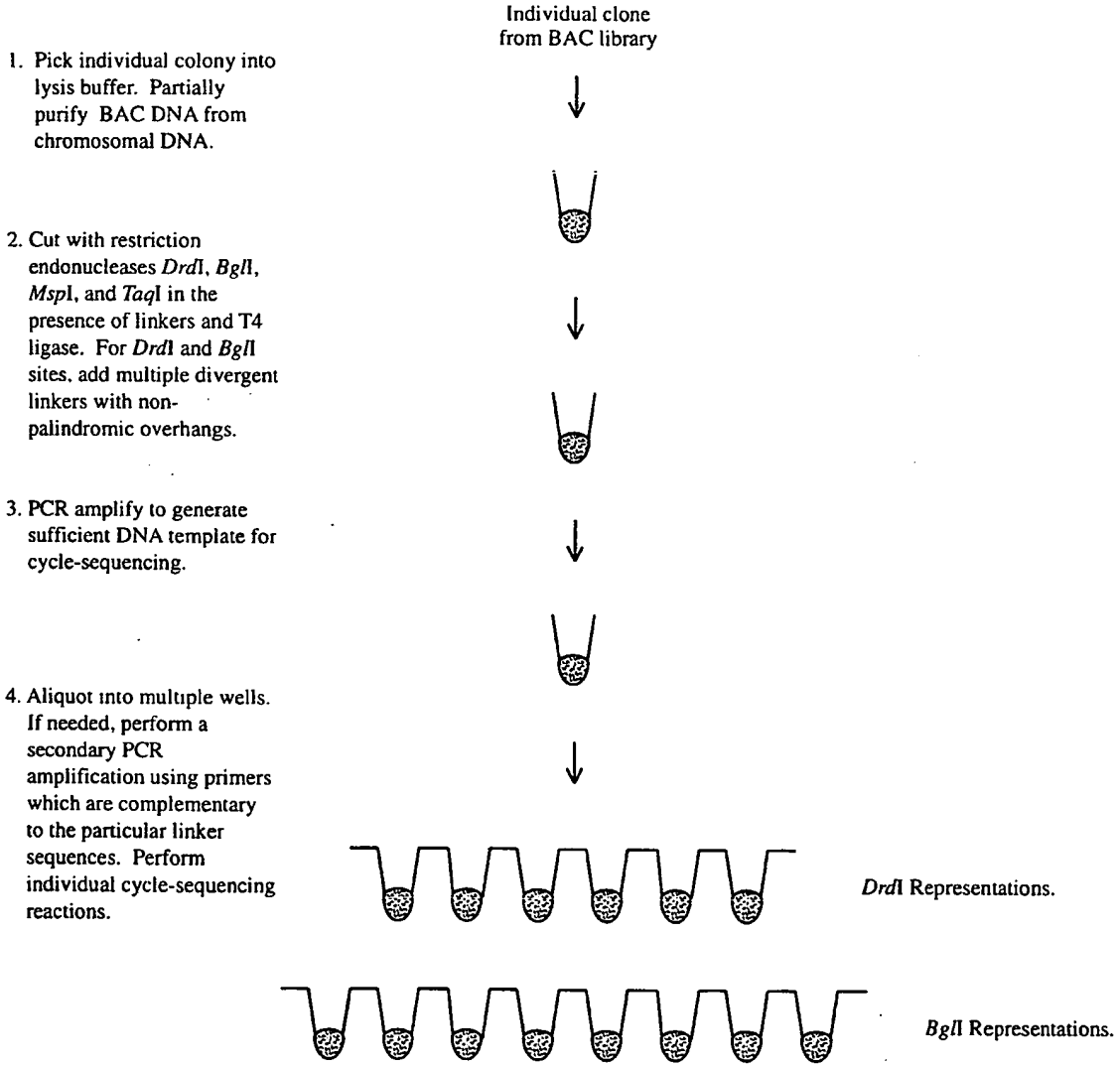
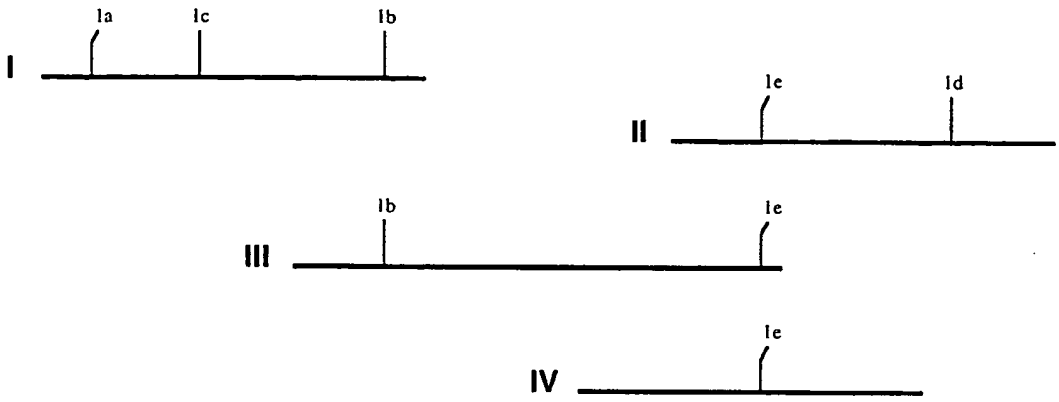


FIG. 37

Overlapping *Drd*I islands in four hypothetical BAC clones: 1 AA overhangs



BAC Clone #	1 = AA	Concordance	1 = AA	Discordance	1 = AA
I	Triplet 1a, 1b, 1c	I & III	Triplet & Doublet (1b)	I & II	1a, b, c ≠ 1d, e
II	Doublet 1d, 1e	II & III	Doublet & Doublet (1e)	I & IV	1a, b, c ≠ 1e
III	Doublet 1b, 1e	III & IV	Doublet & Singlet (1e)		
IV	Singlet 1e	II & IV	Doublet & Singlet (1e)		

Order of *Drd*I islands in four BAC clones.

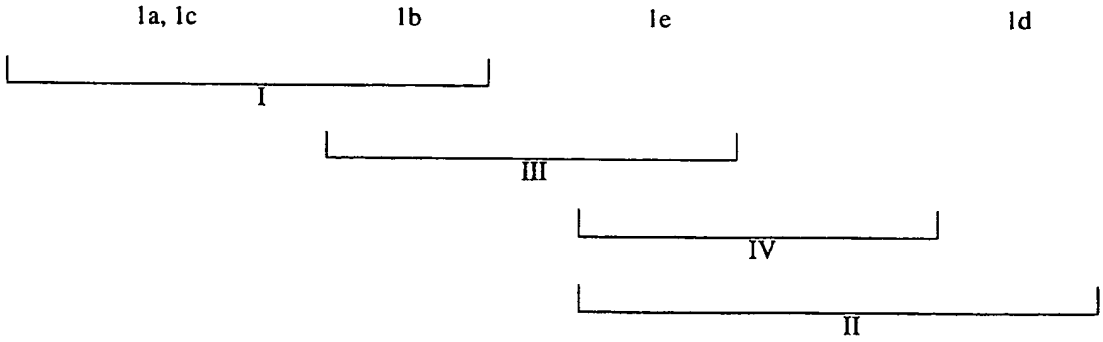
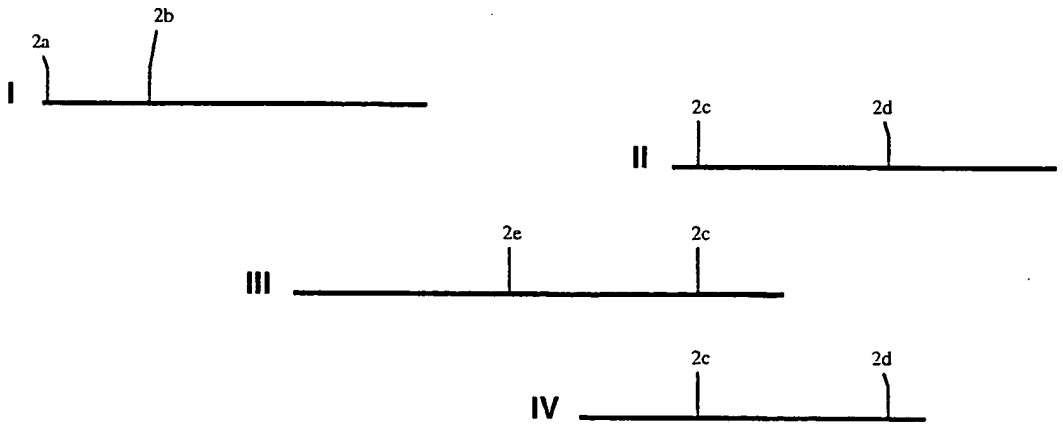


FIG. 38

Overlapping *DrdI* islands in four hypothetical BAC clones: 2 AC overhangs



BAC Clone #	2 = AC	Concordance	2 = AC	Discordance	2 = AC
I	Doublet 2a, 2b	I & III	No overlap	I & II	2a, b ≠ 2c, d
II	Doublet 2c, 2d	II & III	Doublet & Doublet (2c)	I & IV	2a, b ≠ 2c, d
III	Doublet 2c, 2e	III & IV	Doublet & Doublet (2c)		
IV	Doublet 2c, 2d	II & IV	Doublet & Doublet (2c, d)		

Order of *DrdI* islands in four BAC clones.

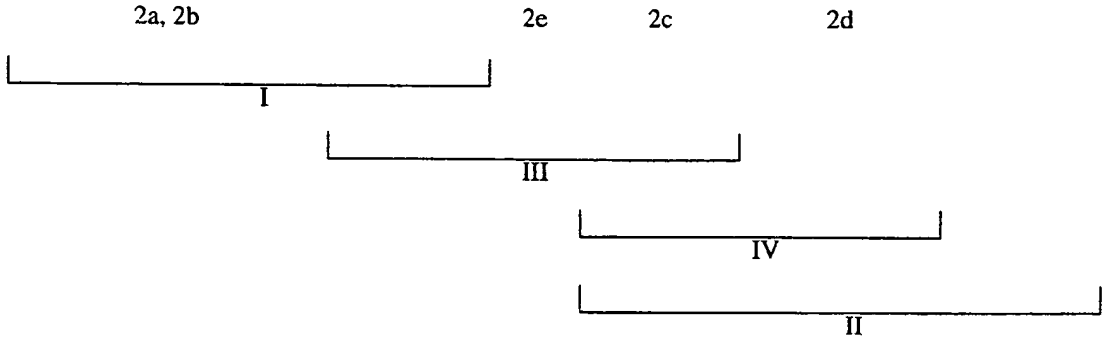
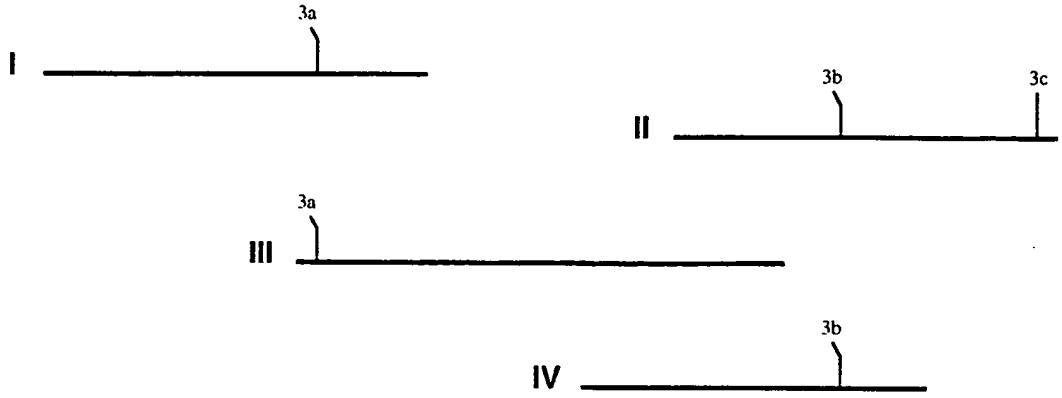


FIG. 39

Overlapping *DrdI* islands in four hypothetical BAC clones: 3 AG overhangs



BAC Clone #	3 = AG	Concordance	3 = AG	Discordance	3 = AG
I	Singlet 3a	I & III	Singlet & Singlet (3a)	I & II	3a ≠ 3b, c
II	Doublet 3b, 3c	II & III	No overlap	I & IV	3a ≠ 3b
III	Singlet 3a	III & IV	No overlap		
IV	Singlet 3b	II & IV	Doublet & Singlet (3b)		

Order of *DrdI* islands in four BAC clones.

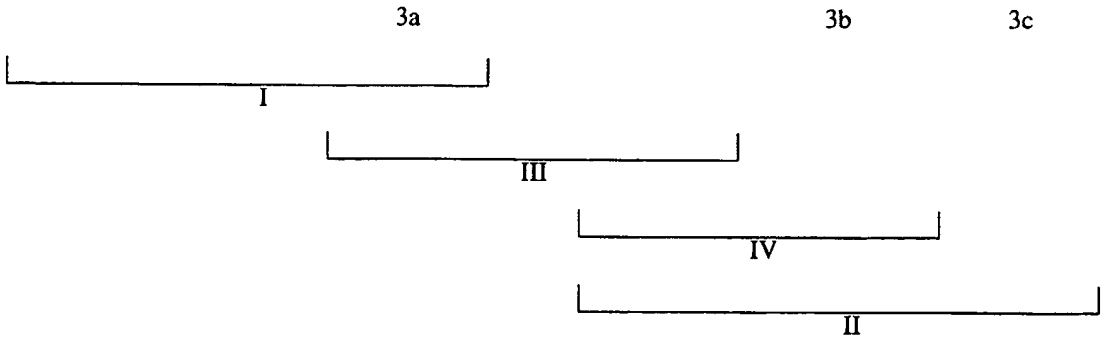
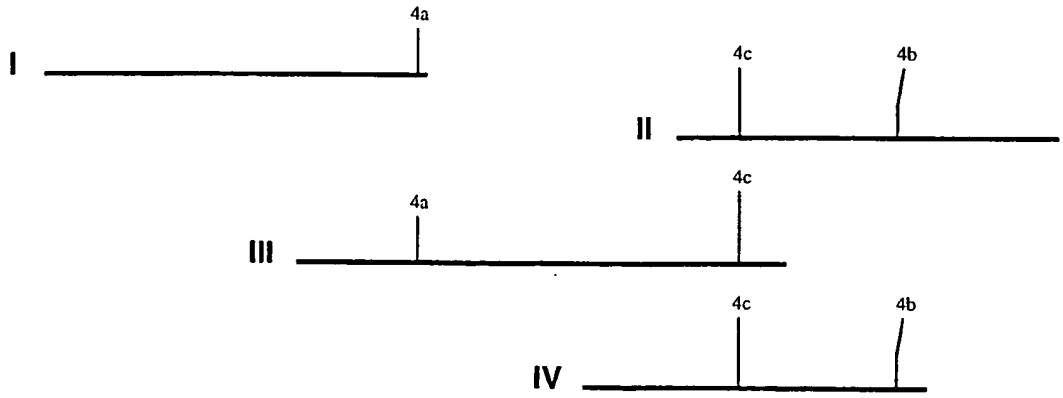


FIG. 40

Overlapping *Drd*I islands in four hypothetical BAC clones: 4 CA overhangs



BAC Clone #	4 = CA	Concordance	4 = CA	Discordance	4 = CA
I	Singlet 4a	I & III	Singlet & Doublet (4a)	I & II	4a ≠ 4b, c
II	Doublet 4b, 4c	II & III	Doublet & Doublet (4c)	I & IV	4a ≠ 4b, c
III	Doublet 4a, 4c	III & IV	Doublet & Doublet (4c)		
IV	Doublet 4b, 4c	II & IV	Doublet & Doublet (4b, c)		

Order of *Drd*I islands in four BAC clones.

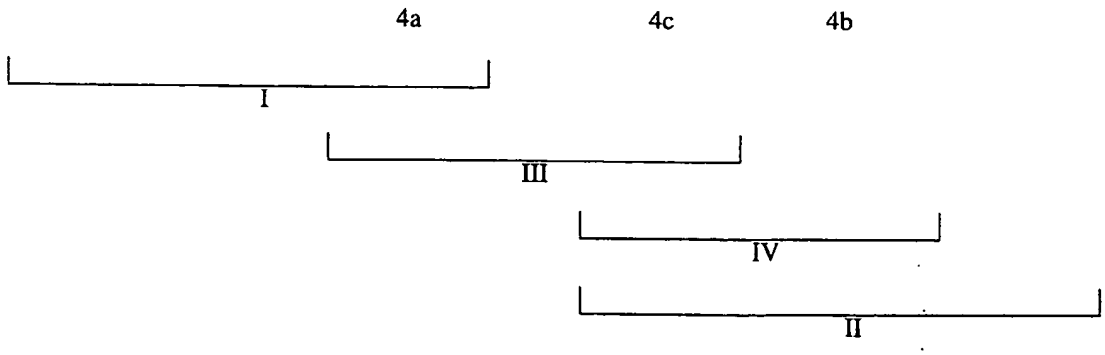
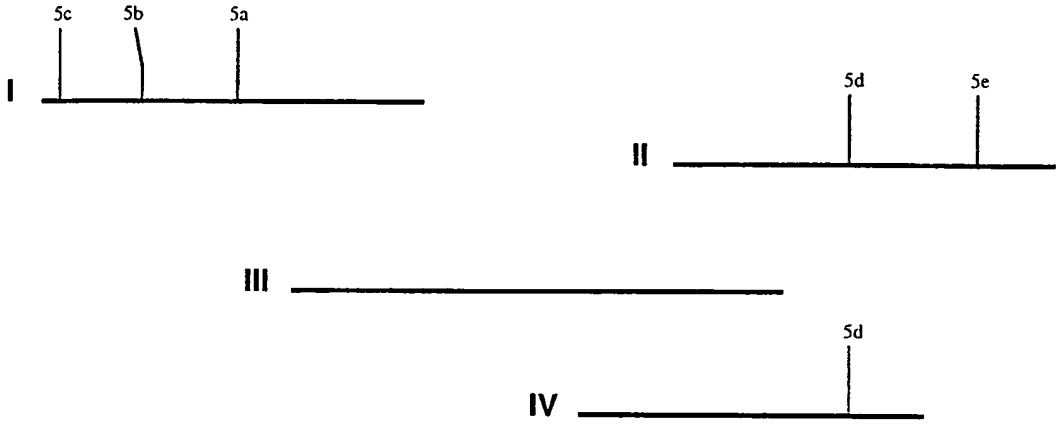


FIG. 41

Overlapping *Drd*I islands in four hypothetical BAC clones: 5GA overhangs



BAC Clone #	5 = GA	Concordance	5 = GA	Discordance	5 = GA
I	Triplet 5a, 5b, 5c	I & III	No overlap	I & II	5a, b, c ≠ 5d, e
II	Doublet 5d, 5e	II & III	No overlap	I & IV	5a, b, c ≠ 5d
III	No sequence	III & IV	No overlap		
IV	Singlet 5d	II & IV	Doublet & Singlet (5d)		

Order of *Drd*I islands in four BAC clones.

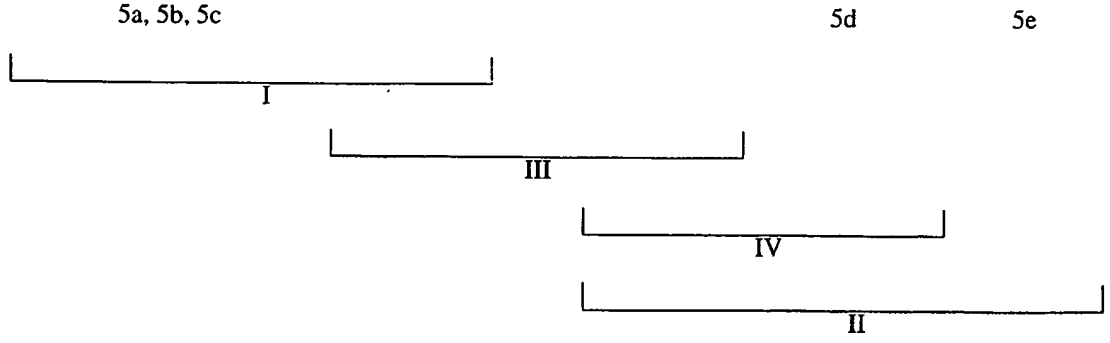
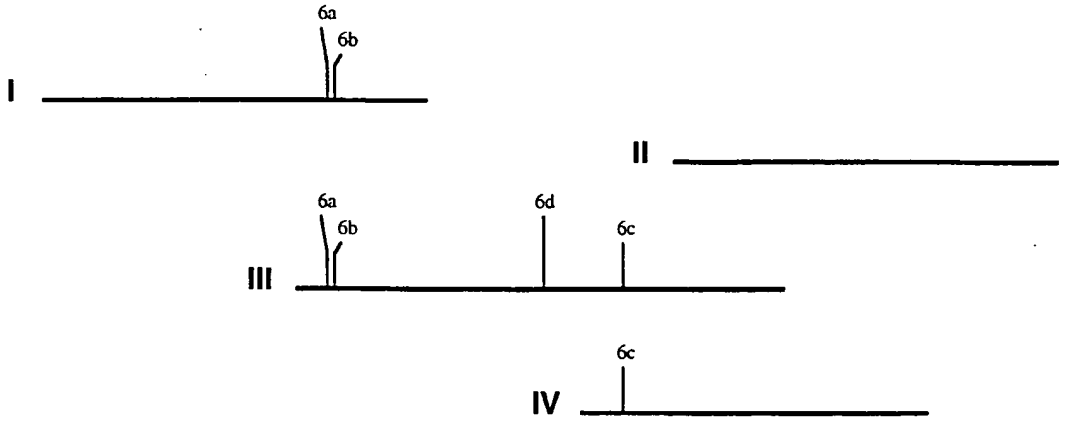


FIG. 42

Overlapping *Drd*I islands in four hypothetical BAC clones: 6 GG overhang



BAC Clone #	6 = GG	Concordance	6 = GG	Discordance	6 = GG
I	Doublet 6a, 6b	I & III	Indeterminant	I & II	-
II	No sequence	II & III	No overlap	I & IV	6a, b ≠ 6c
III	Multiplet (6a, 6b, 6c, 6d)	III & IV	Indeterminant		
IV	Singlet 6c	II & IV	No overlap		

Order of *Drd*I islands in four BAC clones.

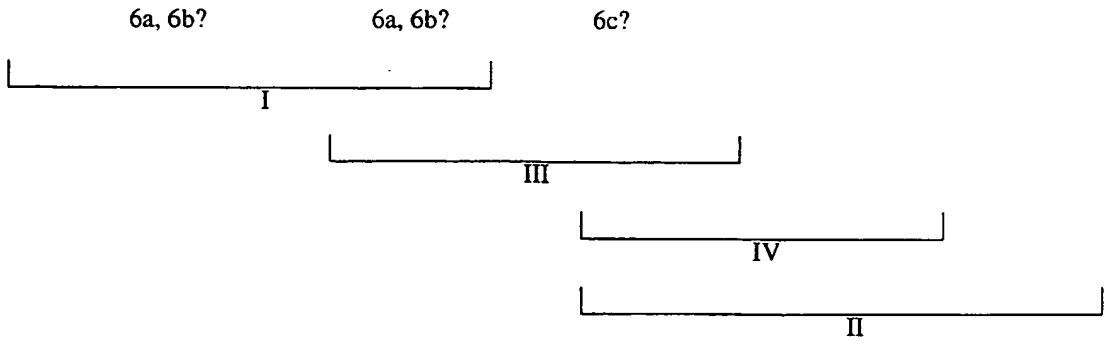
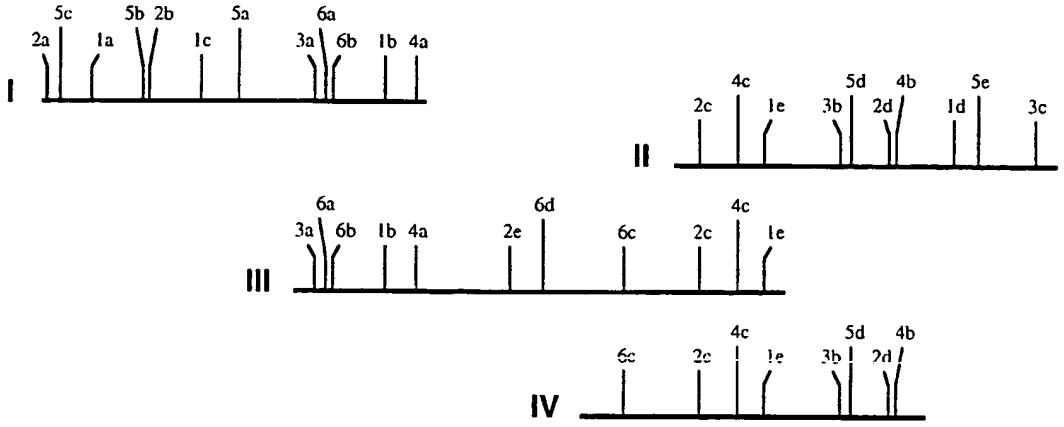


FIG. 43

Overlapping *Drd1* islands in four hypothetical BAC clones



BAC Clone #	1 = AA	2 = AC	3 = AG	4 = CA	5 = GA	6 = GG
I	Triplet 1a, 1b, 1c	Doublet 2a, 2b	Singlet 3a	Singlet 4a	Triplet 5a, 5b, 5c	Doublet 6a, 6b
II	Doublet 1d, 1e	Doublet 2c, 2d	Doublet 3b, 3c	Doublet 4b, 4c	Doublet 5d, 5e	No sequence
III	Doublet 1b, 1e	Doublet 2c, 2e	Singlet 3a	Doublet 4a, 4c	No sequence	Multiplet (6a, 6b, 6c, 6d)
IV	Singlet 1e	Doublet 2c, 2d	Singlet 3b	Doublet 4b, 4c	Singlet 5d	Singlet 6c

Concordance	1 = AA	2 = AC	3 = AG	4 = CA	5 = GA	6 = GG
I & III	Triplet & Doublet (1b)	No overlap	Singlet & Singlet (3a)	Singlet & Doublet (4a)	No overlap	Indeterminant
II & III	Doublet & Doublet (1e)	Doublet & Doublet (2c)	No overlap	Doublet & Doublet (4c)	No overlap	No overlap
III & IV	Doublet & Singlet (1e)	Doublet & Doublet (2c)	No overlap	Doublet & Doublet (4c)	No overlap	Indeterminant
II & IV	Doublet & Singlet (1e)	Doublet & Doublet (2c, d)	Doublet & Singlet (3b)	Doublet & Doublet (4b, c)	Doublet & Singlet (5d)	No overlap
Discordance						
I & II	1a, b, c ≠ 1d, e	2a, b ≠ 2c, d	3a ≠ 3b, c	4a ≠ 4b, c	5a, b, c ≠ 5d, e	-
I & IV	1a, b, c ≠ 1e	2a, b ≠ 2c, d	3a ≠ 3b	4a ≠ 4b, c	5a, b, c ≠ 5d	6a, b ≠ 6c

FIG. 44

Summary of unique and overlapping *Drd*I islands in four hypothetical BAC clones:

Unique I	(1a,c), (2a,b), (5a, b, c)
Overlap I & III	1b, 3a, 4a
Unique II	1d, 5e, 3c
Overlap II & III	1e, 2c, 4c
Overlap II & IV	1e, (2c, d), 3b, (4b, c) 5d
Unique III	2e
Overlap I & III	1b, 3a, 4a
Overlap II & III	1e, 2c, 4c
Overlap III & IV	1e, 2c, 4c
Unique IV	(No unambiguous unique site)
Overlap II & IV	1e, (2c, d), 3b, (4b, c) 5d
Overlap III & IV	1e, 2c, 4c

Order of *Drd*I islands in four BAC clones.

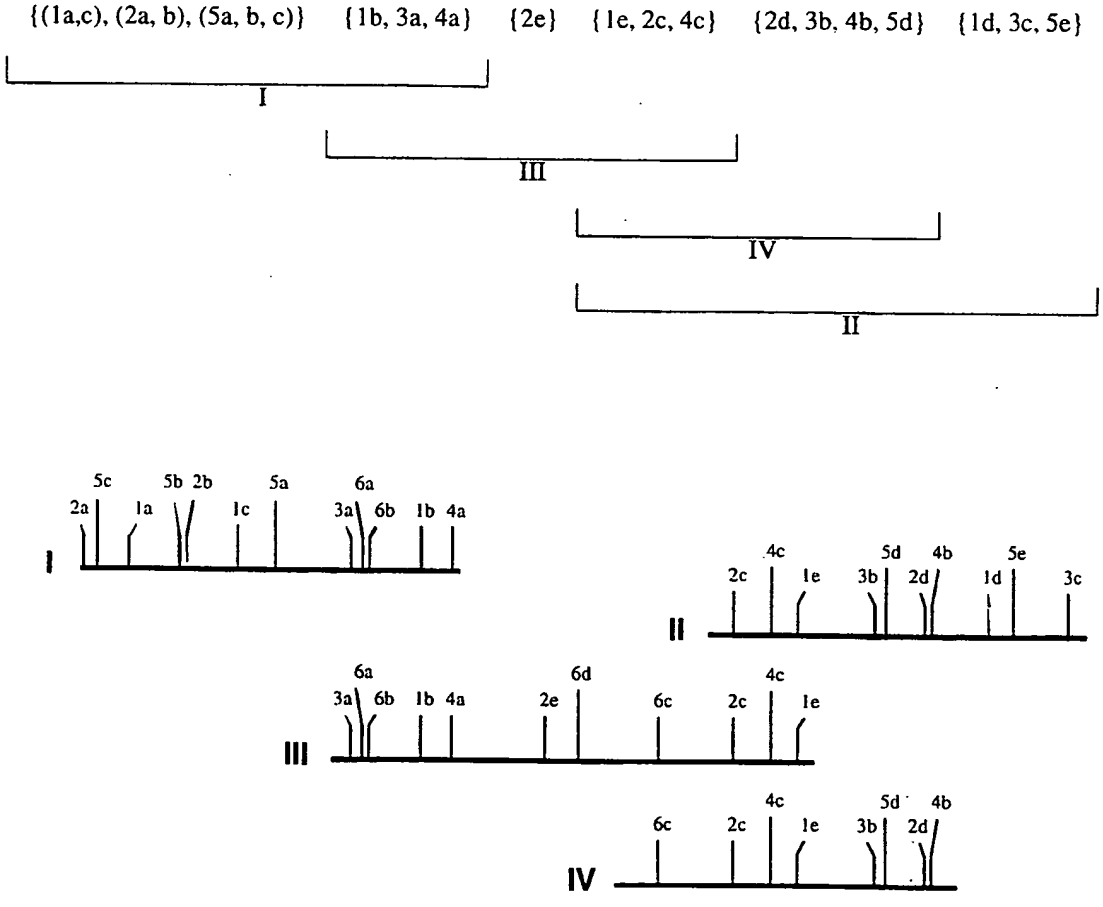


FIG. 45

***DrdI*, *TaqI* and *MspI* sites in overlapping BACs from 7q31**
Contig 1941 (RG253B13, RG013N12, and RG300C03)

DrdI, MspI, TaqI

	AG	AC	CA	GA	AA	GG
RG253B13	546*	502	559*	3,419*	192*	864
	353*	381*			597*	4,918
RG013N12	546*	381*	559*	3,419*	192*	
	353*	1,099	359		597*	
	1,137†		16†		2,040	
					2,328†	
RG300C03	1,1137†	212	16†		2,328†	
		1,008			224	
					1,035	
pBeloBac11			141	360	66	
				691		

	CT	GT	TG	TC	TT	CC
RG253B13	1620*	4497	754*	915*	1794*	3641
	1631*	50*			296*	1866
RG013N12	1620*	50*	754*	915*	1794*	
	1631*	7278	1908	811	296*	
	2077†		183†		525	
					372†	
RG300C03	2077†	282	183†		372†	
					1227	
					1103	
pBeloBac11			127	238	145	
				199		

RG253B13/ RG013N12 = * RG013N12/R RG300C03 = †

FIG. 46

***Drd*I, *Taq*I and *Msp*I sites in overlapping BACs from 7q31**
Contig T002144 (RG022J17, RG067E13, RG011J21, RG022C01, and RG043K06)

DrdI/MspI/TaqI

	AG	AC	CA	GA	AA	GG
RG022J17	1,215*	563		2,977	933	
				77*	2,608	
				142*	71*	
				4,502*	492*	
RG067E13	1,215*	2,001†		77*	71*	
				142*	492*	
				4,502*		
RG011J21		2,001†		8	6,019‡	3,661‡
		699	235			
RG022C01					6,019‡	3,661‡
					2,043**	
RG043K06			2,127	510	2,043**	
			39		5,578	
			4			
pBeloBac11			141	360	66	
				691		

RG022J17/ RG067E13 = * RG067E13/RG011J21 = † RG011J21 / RG022C01 = ‡
RG022C01/ RG043K06 = **

FIG. 46 (cont.)

DrdI/MspI/TaqI

	CT	GT	TG	TC	TT	CC
RG022J17	5335*		1433	328	306	6*
			6190	1427*	2216	
				663*	114*	
				2311*	1470*	
RG067E13	5335*	571†		1427*	114*	6*
				663*	1470*	
				2311*		
RG011J21	544‡	571†	4716	4298		2437‡
		2399	2156			
RG022C01	544‡				5491**	2437‡
RG043K06			19	3213	5491**	
			1510		1981	
			2821			
pBeloBac11			127	238	145	
				199		

RG022J17/ RG067E13 = * RG067E13/RG011J21 = † RG011J21 / RG022C01 = ‡
RG022C01/ RG043K06 = **

FIG. 46 (cont.)

***Drd*I, *Taq*I and *Msp*I sites in overlapping BACs from 7q31**
Contig T002149 (RG343P13, RG205G13, O68P20, and H-133K23)

DrdI/MspI/TaqI

	AG	AC	CA	GA	AA	GG
RG343P13			861		416	
	157*		4		426*	
					52*	
RG205G13	157*	396†			426*	
					52*	
O68P20	825	396†	155	241‡	517	749‡
			1,178		119	
			285			
			2,758			
			1,161‡			
H_133K23	5984		1,161‡	241‡		749‡
	804					
pBeloBac11			141	360	66	
				691		

RG343P13/ RG205G13 = * RG205G13/ O68P20 = † O68P20/ H_133K23 = ‡

FIG. 46 (cont.)

DrdI/MspI/TaqI

	CT	GT	TG	TC	TT	CC
RG343P13	1348		4	246	144	
	58*				110*	
					45*	
RG205G13	58*				110*	
					45*	
O68P20	1146		61	488‡	2438	1567‡
			4573		394	
			1456			
			1774			
			330‡			
H_133K23			330‡	488‡		1567‡
				3335		
				1181		
pBeloBac11			127	238	145	
				199		

RG343P13/ RG205G13 = * RG205G13/ O68P20 = † O68P20/ H_133K23 = ‡

FIG. 46 (cont.)

***Drd*I and *Mse*I sites in overlapping BACs from 7q31**
Contig 1941 (RG253B13, RG013N12, and RG300C03)

Drd/MseI

	AG	AC	CA	GA	AA	GG
RG253B13	546*	203	294	36*	687*	32
	142*	47*				935
RG013N12	546*	47*	404	36*	687*	
	142*	195	277†	103	325	
	39†				24†	
RG300C03	39†	132	277†		24†	
		379			190	
					14	
pBeloBac11			87	484	344	
				136		

RG253B13/ RG013N12 = * RG013N12/R RG300C03 = †

FIG. 47

***DrdI*, *TaqI* and *MspI* sites in overlapping BACs from 7q31**
Contig T002144 (RG022J17, RG067E13, RG011J21, RG022C01, and RG043K06)

DrdI/MseI

	AG	AC	CA	GA	AA	GG
RG022J17	338*	109	134	38	19	55*
				586*	148	
				77*	273*	
				17*		
RG067E13	338*	71†		586*	273*	55*
				77*		
				17*		
RG011J21	92‡	71†	276	214	48‡	42‡
		30	248			
RG022C01	92‡				48‡	42‡
RG043K06			550	59	80	
			77			
			32			
pBeloBac11			87	484	344	
				136		

RG022J17/ RG067E13 = * RG067E13/RG011J21 = † RG011J21 / RG022C01 = ‡
RG022C01/ RG043K06 = **

FIG. 47 (cont.)

DrdI/MseI

	CT	GT	TG	TC	TT	CC
RG022J17	368*		329	70	33	163*
			186	84*	182	
				36*	296*	
				57*	59*	
RG067E13	368*	161†		84*	296*	163*
				36*	59*	
				57*		
RG011J21	41‡	161†	45	49	270‡	101‡
		46	30			
RG022C01	41‡				270‡	101‡
					29**	
RG043K06			76	12	29**	
			35		65	
			51			
pBeloBac11			46	21	420	
				115		

RG022J17/ RG067E13 = * RG067E13/RG011J21 = † RG011J21 / RG022C01 = ‡
RG022C01/ RG043K06 = **

FIG. 47 (cont.)

***Drd*I and *Mse*I sites in overlapping BACs from 7q31.**
Contig T002149 (RG343P13, RG205G13, O68P20, and H-133K23)

DrdI/MseI

	AG	AC	CA	GA	AA	GG
RG343P13	1076*		597		102	
			184		648*	
					286*	
RG205G13	1076*	89†			648*	
					286*	
O68P20	59	89†	134	21‡	26	168‡
			62		63	
			22			
			206‡			
H_133K23	155		206‡	21‡		168‡
	36					
pBeloBac11			87	484	344	
				136		

RG343P13/ RG205G13 = * RG205G13/ O68P20 = † O68P20/ H_133K23 = ‡

FIG. 47 (cont.)

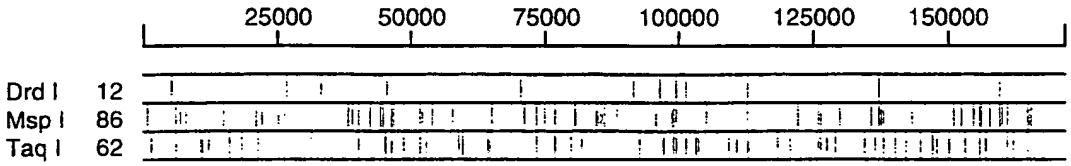
DrdI/MseI

	CT	GT	TG	TC	TT	CC
RG343P13	41		129	73	53	
	53*		213		489*	
RG205G13	53*	51†			489*	
O68P20	21	51†	25	92‡	307	78‡
			48		183	
			23			
			62			
			227‡			
H_133K23			227‡	92‡		78‡
				31		
				342		
pBeloBac11			46	21	420	
				115		

RG343P13/ RG205G13 = * RG205G13/ O68P20 = † O68P20/ H_133K23 = ‡

FIG. 47 (cont.)

RG253B13, 7q31 Met Oncogene
12 *Drd*I, 86 *Msp*I, and 62 *Taq*I Sites in 171,905 bp



Sequence : BAC RG253B13.seq (1 > 171905) 160 Cut Sites

5385	Drd I	81884	Taq I	99370	Taq I
6249	Taq I	84572	Msp I	99513	Msp I
6381	Msp I	84594	Msp I	99628	Drd I
		84831	Msp I		
21540	Taq I	85041	Msp I	101248	Taq I
22165	Msp I	85105	Msp I	101440	Drd I
25228	Msp I	85155	Msp I		
26363	Msp I	85212	Msp I	113048	Drd I
26871	Drd I	85523	Msp I	113429	Taq I
		85538	Msp I	118458	Taq I
33306	Drd I	85569	Msp I	120734	Taq I
38218	Msp I	85625	Msp I	122429	Msp I
39316	Msp I	85655	Msp I		
40280	Msp I	85670	Msp I	135890	Msp I
40344	Msp I	86173	Msp I	136580	Taq I
40389	Taq I	88256	Msp I	137177	Drd I
		91681	Drd I		
45534	Drd I			159685	Drd I
		96506	Drd I	160038	Msp I
70528	Drd I	97059	Taq I	160434	Msp I
		98602	Msp I	161212	Taq I

For AA, AC, AG, CA, GA, and GG overhangs

*Drd*I#	Location	Overhang	Complement	Nearest *Msp*I	Nearest *Taq*I	Fragment Length
1.	5,379	GG*	CC	6,381	6,249	864
2.	26,865	GT	AC*	26,363	21,540	502
3.	33,300	GG*	CC	38,218	40,389	4,918
4.	45,528	AT	AT			
5.	70,522	AT	AT			
6.	91,675	TC	GA*	88,256	81,884	3,419
7.	96,500	CA*	TG	98,602	97,059	559
8.	99,622	CT	AG*	99513	99,370	115
9.	101,434	TT	AA*		101,248	192
10.	113,042	AC*	GT	122,429	113,429	381
11.	137,171	TT	AA*	135,890	136,580	597
12.	159,679	AG*	CT	160,038	161,212	353

* To obtain sequence information on AA, AC, AG, CA, GA, or GG overhangs in the sense direction, the *Drd*I island is amplified using a downstream *Msp*I or *Taq*I site. For such two base sequences on the complementary strand, the *Drd*I island is amplified using an upstream *Msp*I or *Taq*I site.

FIG. 48

PCR amplification of *Drd*I representation for shotgun cloning and generating mapped SNPs.

1. Cut genomic DNAs with *Msp*I, *Taq*I and *Drd*I in the presence of linkers and T4 ligase. Linker for *Drd*I site is phosphorylated, methylated at internal *Xma*I site, and contains a 3' AA overhang. Linker for *Msp*I site is not phosphorylated, methylated at internal *Xho*I site, and contains a bubble. Biochemical selection assures that most sites contain linkers.

2. Inactivate T4 ligase and restriction endonucleases at 95°C for 5 min. PCR amplify using unmethylated primers, dNTPs, and *Taq* polymerase. Conditions are optimized to amplify about 35,000 fragments at high yield while minimizing bias in the representation.

3. Cut PCR products with *Xma*I and *Xho*I, separate mixed fragments on an agarose gel, select and purify 200-1,000 bp fragments and clone into the corresponding sites of a standard vector. Sequence inserts to build mapped SNP database.

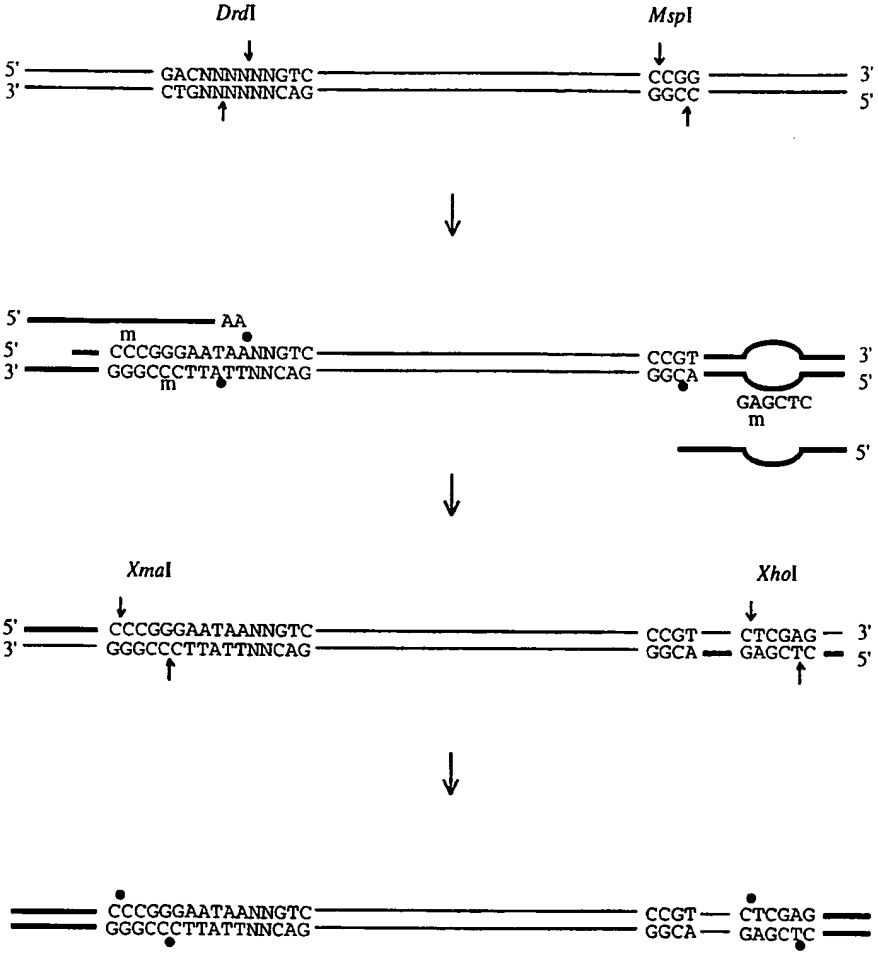


FIG. 49

PCR amplification of *Drd*I representation for shotgun cloning and generating mapped SNPs.

1. Cut genomic DNAs with *Msp*I, *Taq*I and *Drd*I in the presence of linkers and T4 ligase. Linker for *Drd*I site is phosphorylated, methylated at internal *Xma*I site, and contains a 3' AA overhang. Linker for *Msp*I site is phosphorylated, 3' blocked, methylated at internal *Xho*I site, and contains a bubble. Biochemical selection assures that most sites contain linkers.

2. Inactivate T4 ligase and restriction endonucleases at 95°C for 5 min. PCR amplify using unmethylated primers, dNTPs, and *Taq* polymerase. Conditions are optimized to amplify about 35,000 fragments at high yield while minimizing bias in the representation.

3. Cut PCR products with *Xma*I and *Xho*I, separate mixed fragments on an agarose gel, select and purify 200-1,000 bp fragments and clone into the corresponding sites of a standard vector. Sequence inserts to build mapped SNP database.

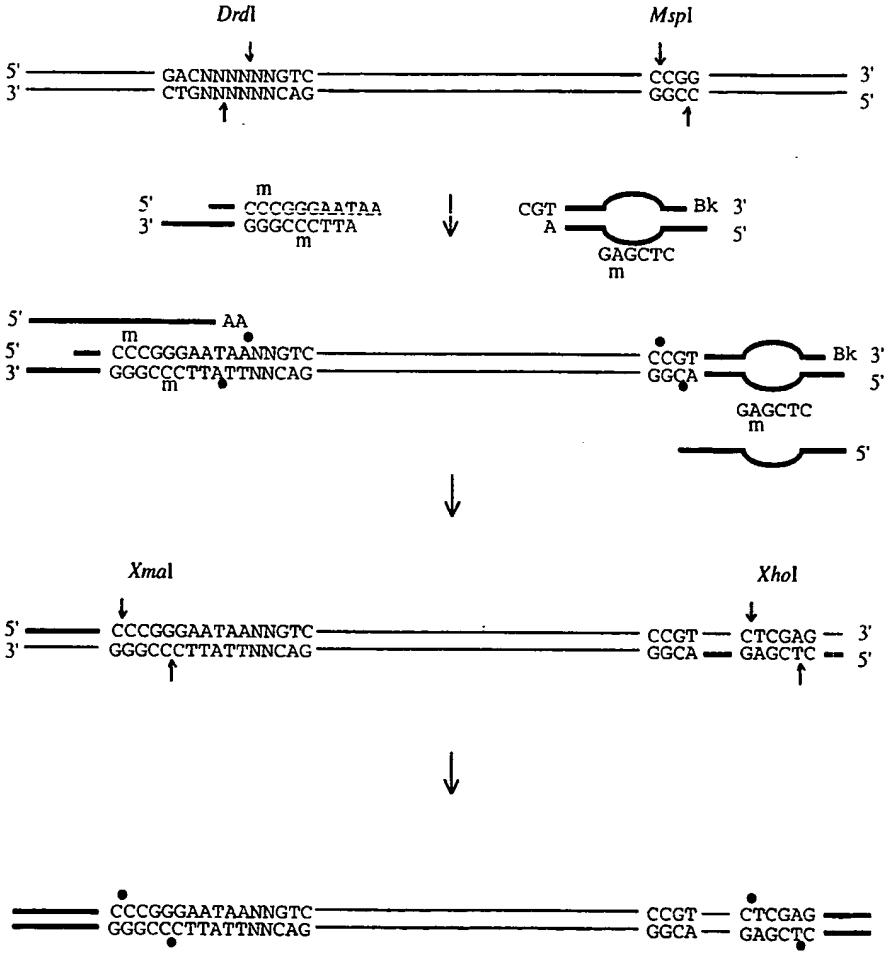


FIG. 49A

PCR amplification of *Drd*I representation for high-throughput SNP detection.

1. Cut genomic DNA with *Msp*I, *Taq*I and *Drd*I in the presence of linkers and T4 ligase. Linker for *Drd*I site is phosphorylated and contains a 3' AA overhang. Linker for *Msp*I site is not phosphorylated, and contains a bubble. Biochemical selection assures that most sites contain linkers.

2. Inactivate T4 ligase and restriction endonucleases at 95°C for 5 min. PCR amplify using *Drd*I primer containing a 3' AAC overhang, dNTPs, and *Taq* polymerase. Conditions are optimized to amplify about 9,000 fragments at high yield while minimizing bias in the representation.

3. Add LDR primers and thermostable ligase to simultaneously detect SNPs at multiple loci. In (A) the common LDR primer contains zip-code Z1, the discriminating primers contain fluorescent labels F1 and F2, after array capture, ratio of F1/F2 determines presence of allele or allele imbalance. In (B) the common LDR primer contains fluorescent label F, the discriminating primers contain zip-codes Z2 and Z3, after array capture, ratio of fluorescence at Z2 and Z3 determines presence of allele or allele imbalance.

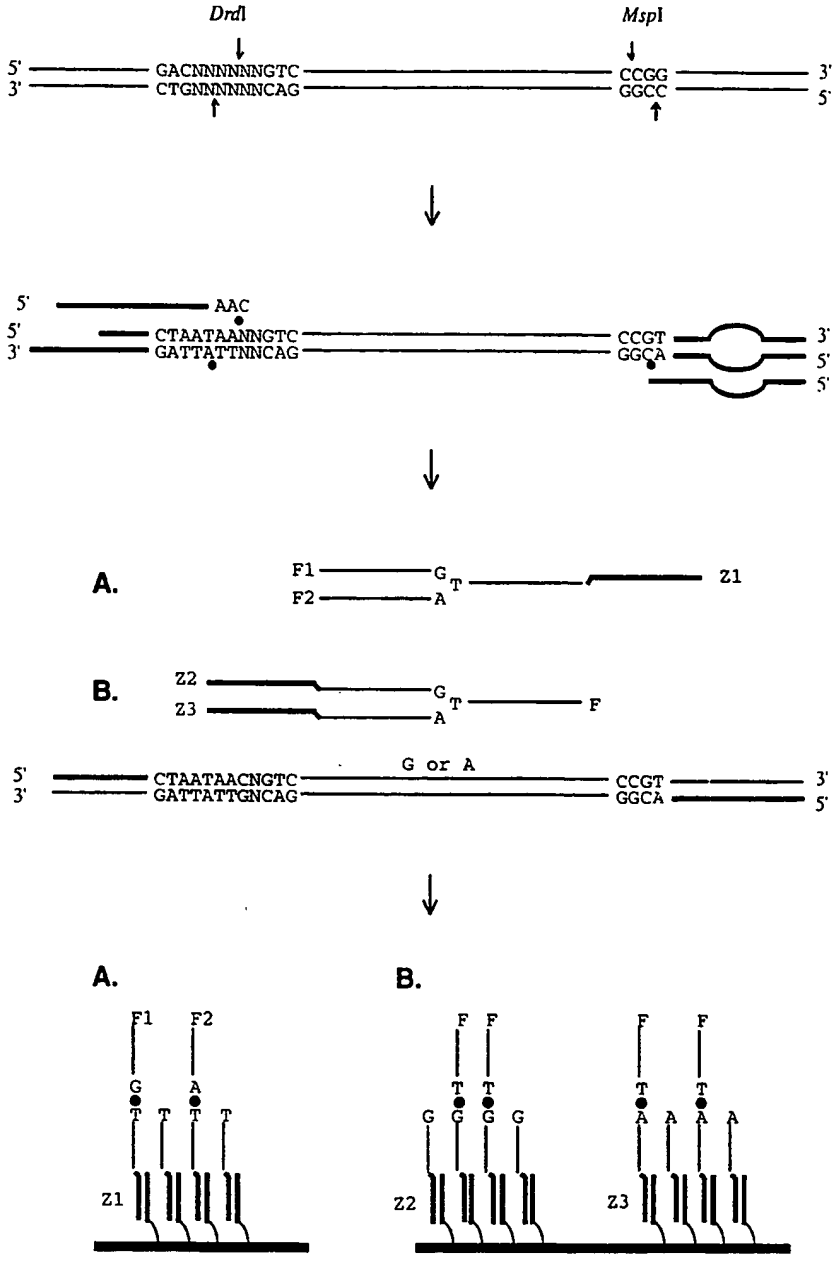


FIG. 50

PCR amplification of *DrdI* representation for high-throughput SNP detection.

1. Cut genomic DNA with *MspI*, *TaqI* and *DrdI* in the presence of linkers and T4 ligase. Linker for *DrdI* site is phosphorylated and contains a 3' AA overhang. Linker for *MspI* site is phosphorylated, 3' blocked and contains a bubble. Biochemical selection assures that most sites contain linkers.

2. Inactivate T4 ligase and restriction endonucleases at 95°C for 5 min. PCR amplify using *DrdI* primer containing a 3' AAC overhang, dNTPs, and *Taq* polymerase. Conditions are optimized to amplify about 9,000 fragments at high yield while minimizing bias in the representation.

3. Add LDR primers and thermostable ligase to simultaneously detect SNPs at multiple loci. In (A) the common LDR primer contains zip-code Z1, the discriminating primers contain fluorescent labels F1 and F2, after array capture, ratio of F1/F2 determines presence of allele or allele imbalance. In (B) the common LDR primer contains fluorescent label F, the discriminating primers contain zip-codes Z2 and Z3, after array capture, ratio of fluorescence at Z2 and Z3 determines presence of allele or allele imbalance.

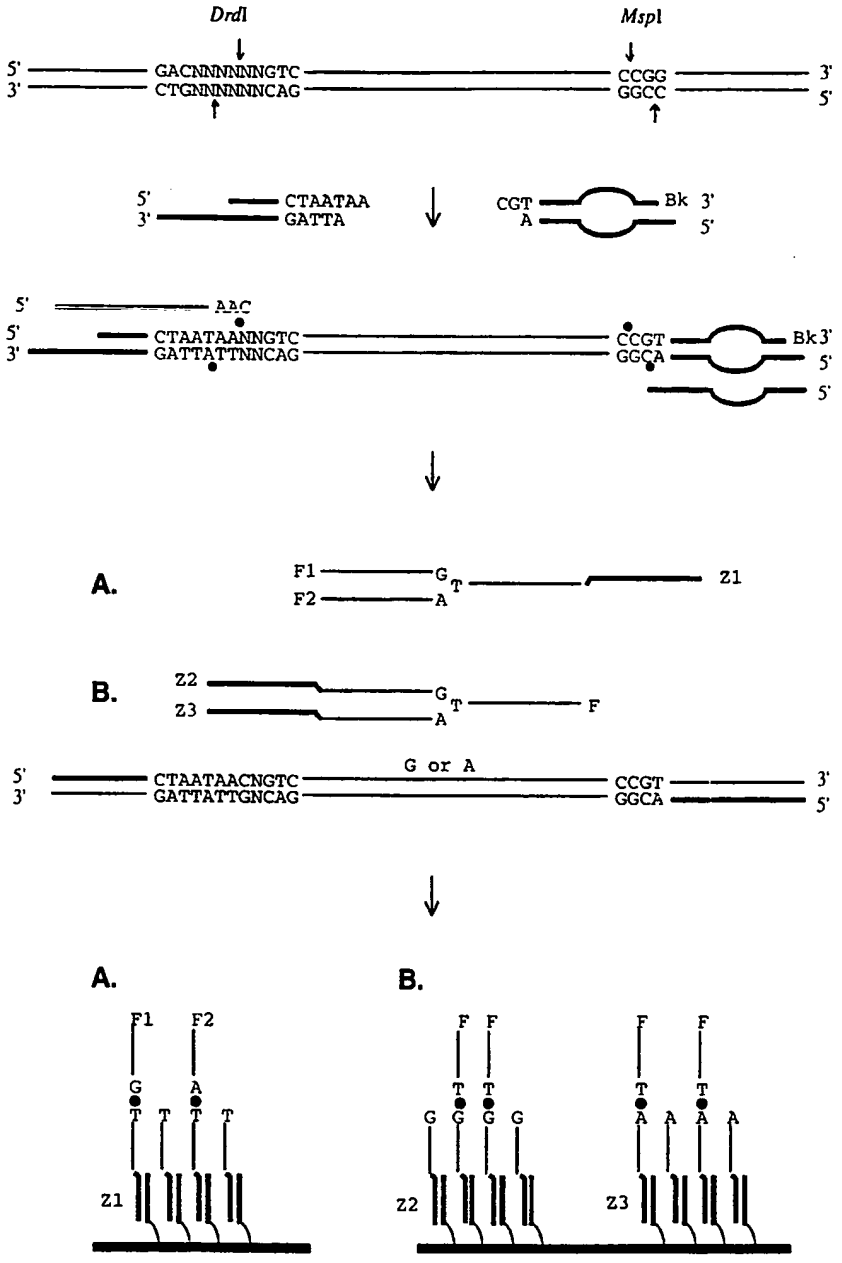


FIG. 50A

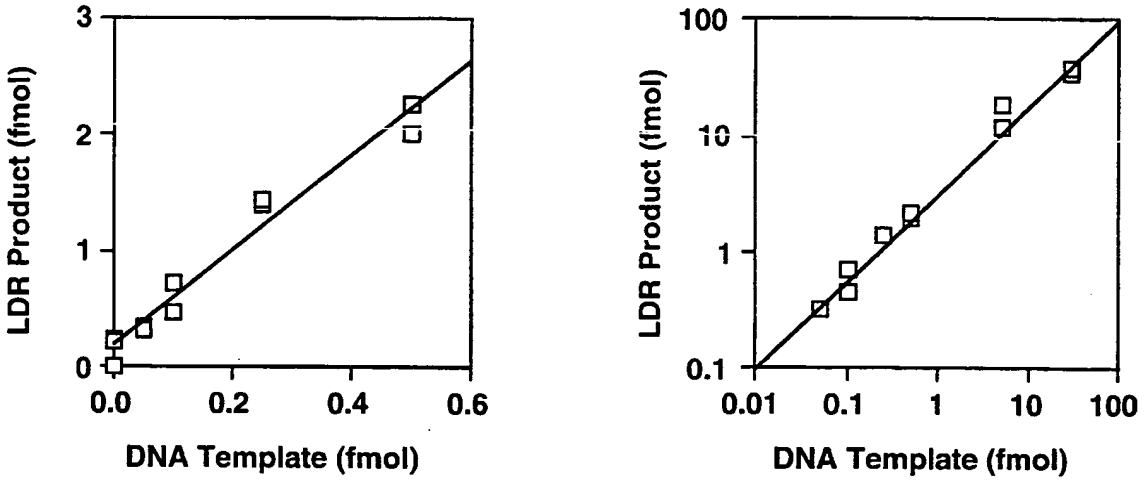

LDR Product (fmol)
3
2
1
0
0.0
0.2
0.4
0.6
DNA Template (fmol)
LDR Product (fmol)
100
10
1
0.1
0.01
0.1
1
10
100
DNA Template (fmol)

FIG. 51

PCR/LDR with Addressable Array Capture

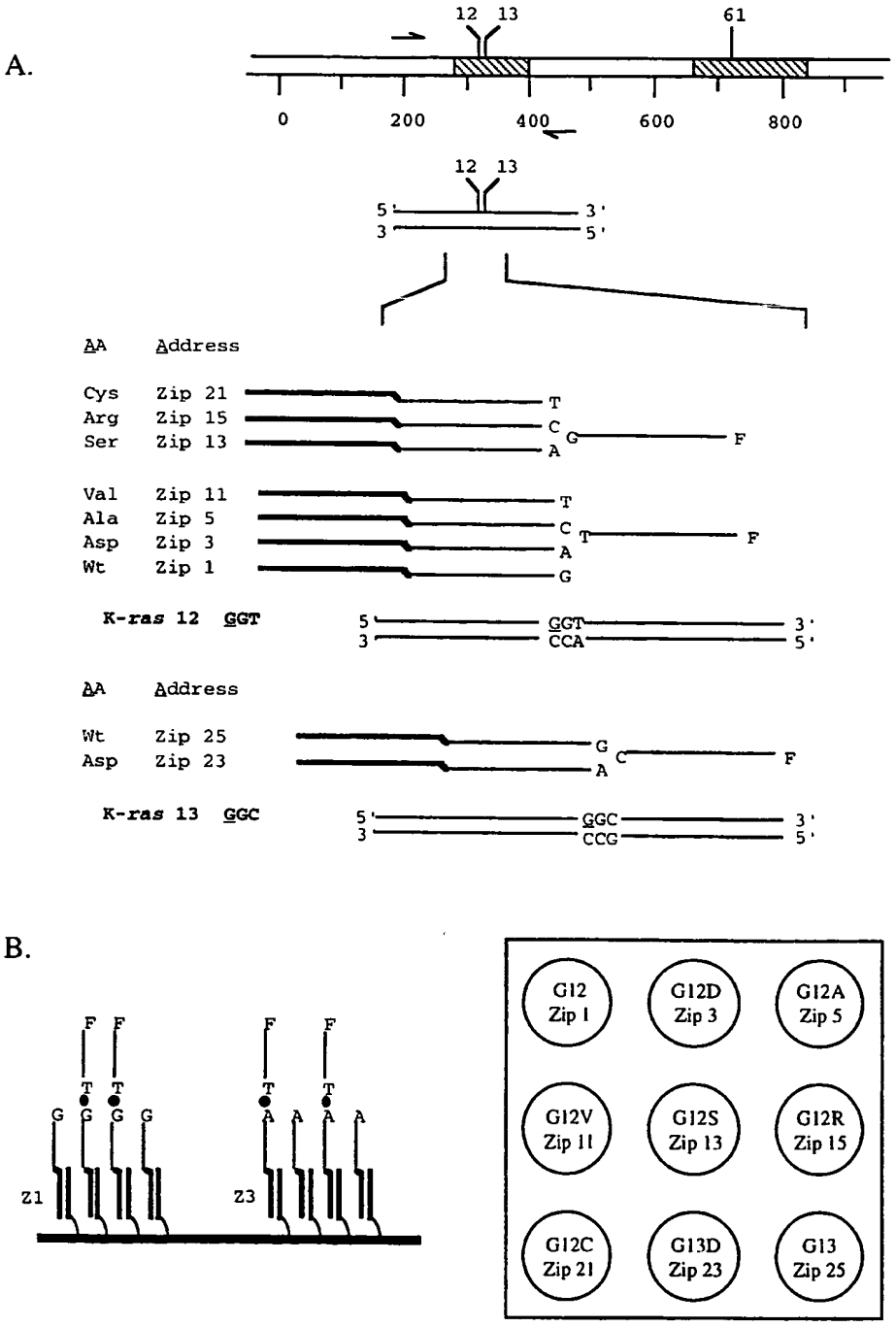


FIG. 52

SUBSTITUTE SHEET (RULE 26)

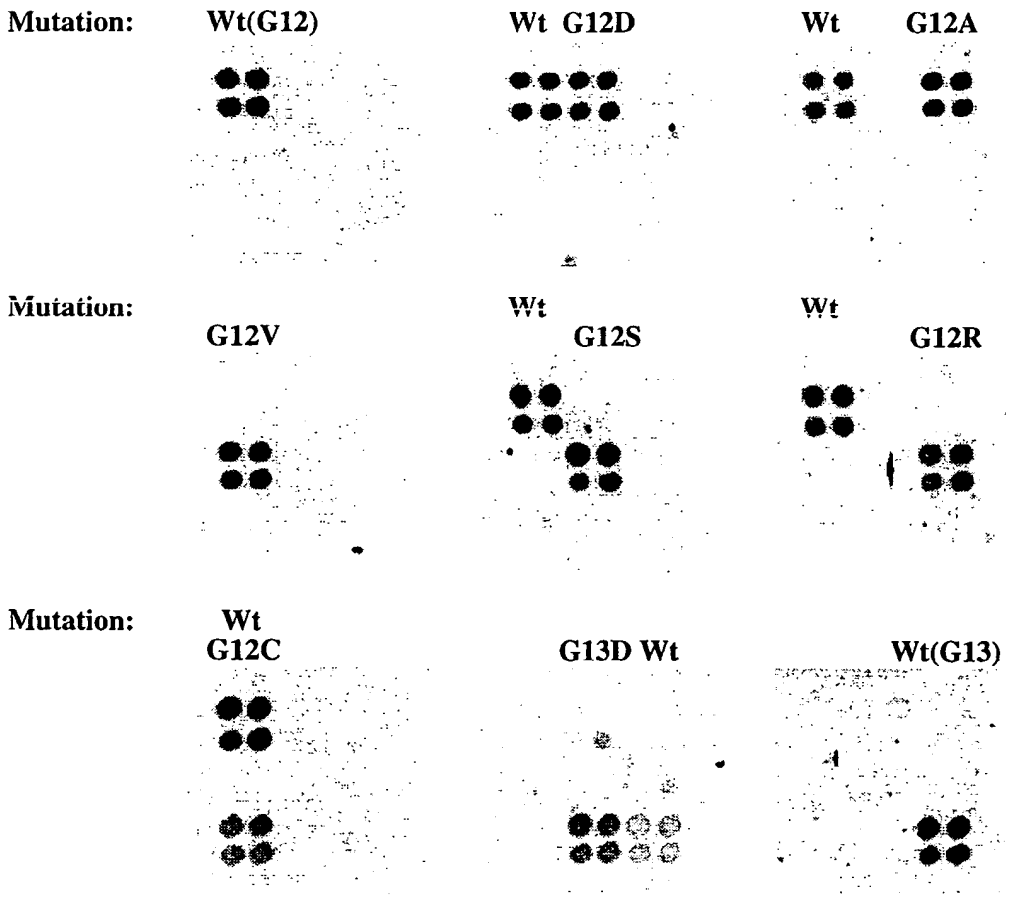

Mutation:
Wt(G12)
Wt G12D
Wt G12A
Mutation:
G12V
Wt
G12S
Wt
G12R
Mutation:
Wt
G12C
G13D Wt
Wt(G13)

FIG. 53

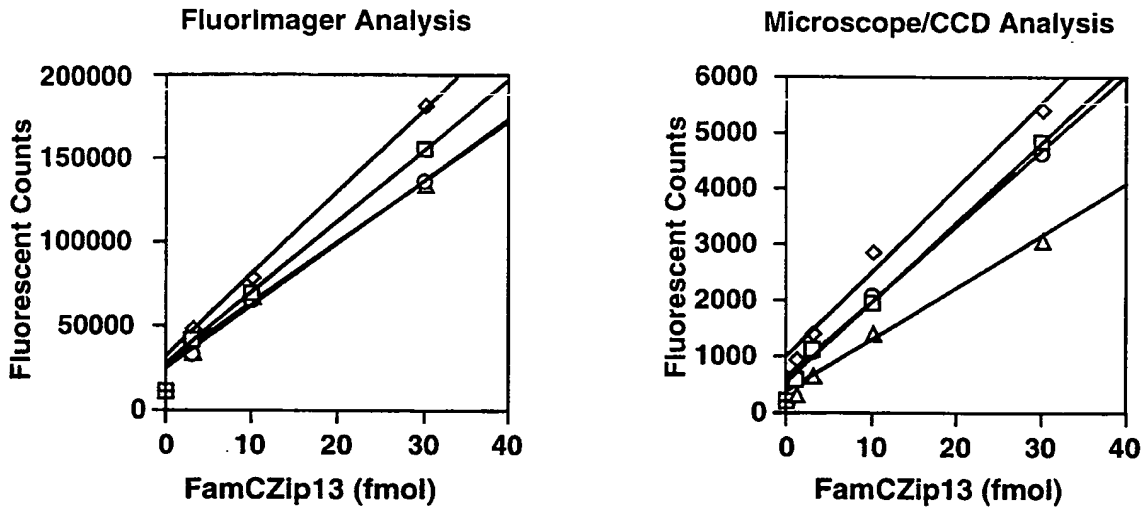
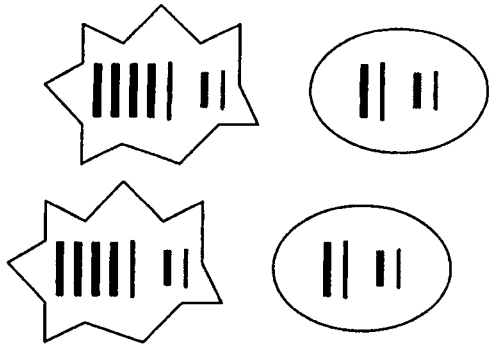

FluorImager Analysis
Fluorescent Counts
200000
150000
100000
50000
0
0
10
20
30
40
FamCZip13 (fmol)
Microscope/CCD Analysis
Fluorescent Counts
6000
5000
4000
3000
2000
1000
0
0
10
20
30
40
FamCZip13 (fmol)

FIG. 54

Amplification
(with 50% stromal contamination)



Ratio of alleles:	Tumor Sample	Normal Sample
:	10 / 4 = 2.5	4 / 4 = 1.0
:	4 / 4 = 1.0	4 / 4 = 1.0

Ratio of Tumor to control gene / normal to control gene:

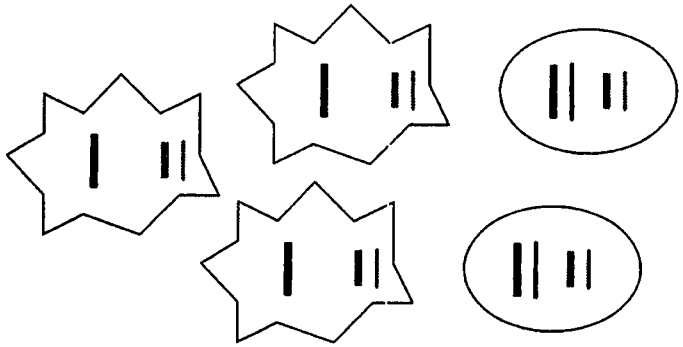
: / : 14 / 8 / 8 / 8 = 1.75

Ratio of Tumor to control allele / normal to control allele:

: / : 10 / 4 / 4 / 4 = 2.5

: / : 4 / 4 / 4 / 4 = 1.0

Loss of Heterozygosity
(with 40% stromal contamination)



Ratio of alleles:	Tumor Sample	Normal Sample
:	5 / 2 = 2.5	5 / 5 = 1.0
:	5 / 5 = 1.0	5 / 5 = 1.0

Ratio of Tumor to control gene / normal to control gene:

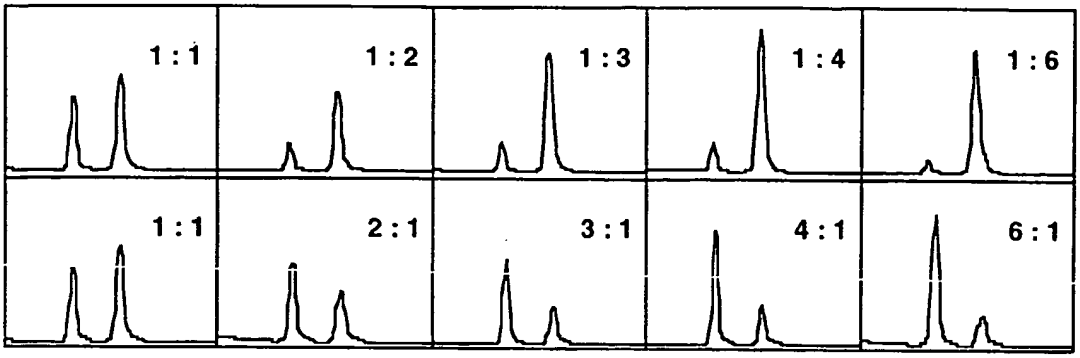
: / : 7 / 10 / 10 / 10 = 0.70

Ratio of Tumor to control allele / normal to control allele:

: / : 5 / 5 / 5 / 5 = 1.0

: / : 2 / 5 / 5 / 5 = 0.4

FIG. 55

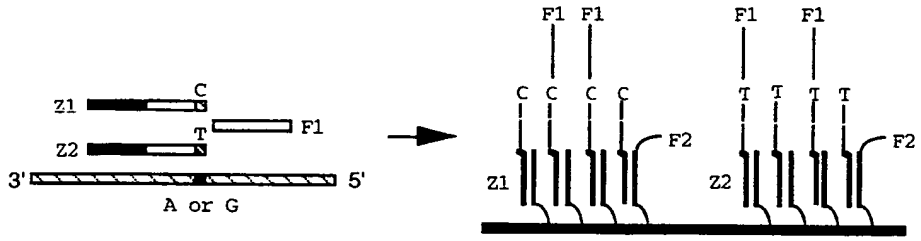


Ratio of Normal to Mutant Template	LDR Product (fmol)		Ratio of LDR Products	
	Normal	Mutant	Absolute	Normalized
1:1	32.2	51.7	0.62	1 : 1.0
1:2	11.8	41.9	0.28	1 : 2.2
1:3	13.7	64.2	0.21	1 : 3.0
1:4	12.8	78.4	0.16	1 : 3.9
1:6	6.5	70.2	0.09	1 : 6.7
1:1	32.2	51.7	0.62	1.0 : 1
2:1	41.6	33.1	1.26	2.0 : 1
3:1	34.1	18.5	1.84	3.0 : 1
4:1	42.7	18.1	2.36	3.8 : 1
6:1	64.4	18.4	3.50	5.7 : 1

FIG. 56

PCR/LDR with Addressable Array Capture

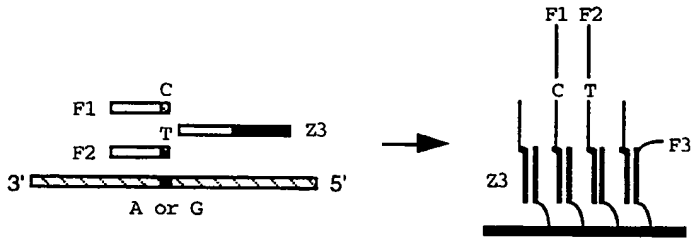
A.



LDR reaction: Zip codes on discriminating primers.

Heterozygous: C and T alleles.

B.



LDR reaction: Zip code on common primer.

Heterozygous: C and T alleles.

FIG. 57

?/SNUPE with Addressable Array Capture

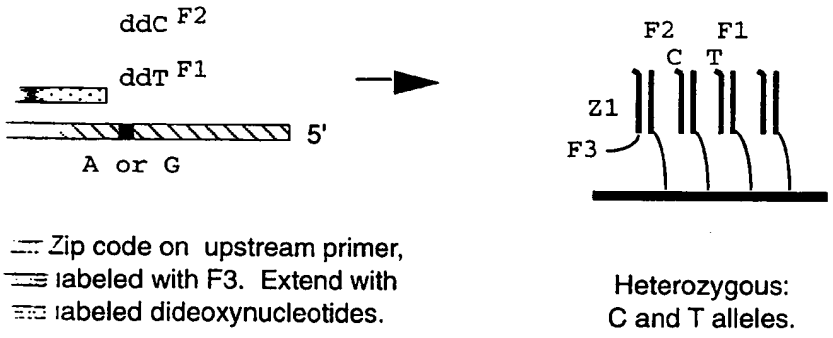
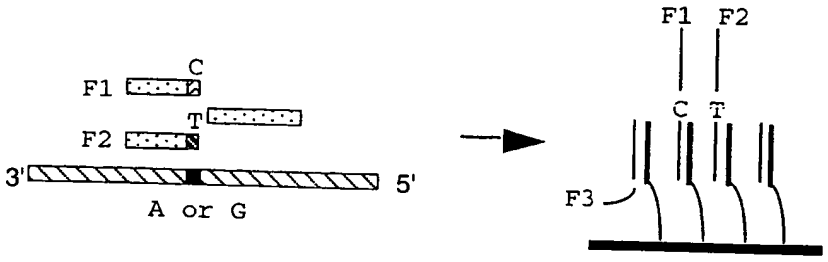


FIG. 58

PCR/LDR with Gene Array Capture

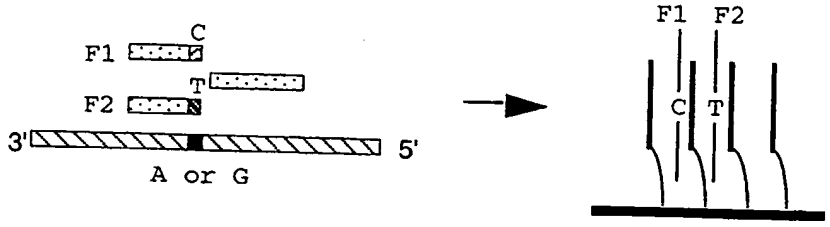
A.



LDR reaction: Small percentage of common primer labeled with F3.

Heterozygous: C and T alleles.

B.



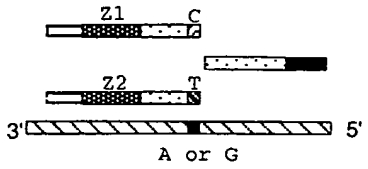
LDR reaction: Product is complementary to array

Heterozygous: C and T alleles.

FIG. 59

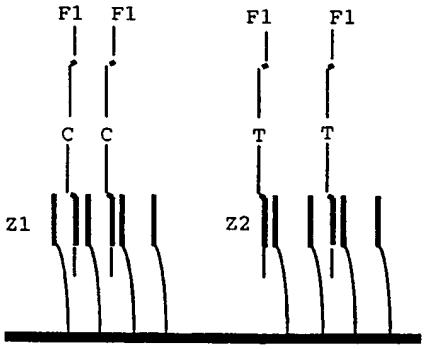
LDR/PCR with Addressable Array Capture

1. LDR reaction: Universal primer and unique Zip codes on 5' side of discriminating primers, universal primer on 3' side of common primer.



2. PCR reaction: Universal primers amplify multiplex LDR products simultaneously. One primer is fluorescently labeled.

3. Capture: Fluorescently labeled products are captured on addressable array at unique zipcode sequences.



Heterozygous:
C and T alleles.

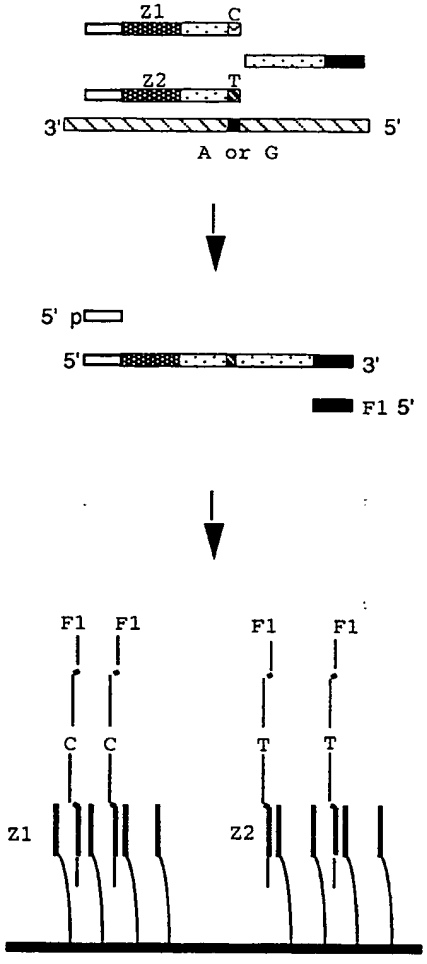
FIG. 60

LDR/PCR with Addressable Array Capture

1. LDR reaction: Universal primer and unique Zip codes on 5' side of discriminating primers, universal primer on 3' side of common primer.

2. PCR reaction: Universal primers amplify multiplex LDR products simultaneously. One primer is fluorescently labeled, while the other contains a 5' phosphate. After PCR amplification, the phosphorylated strand is digested with lambda exonuclease leaving fluorescently labeled single-stranded DNA.

3. Capture: Fluorescently labeled products are captured on addressable array at unique zipcode sequences.

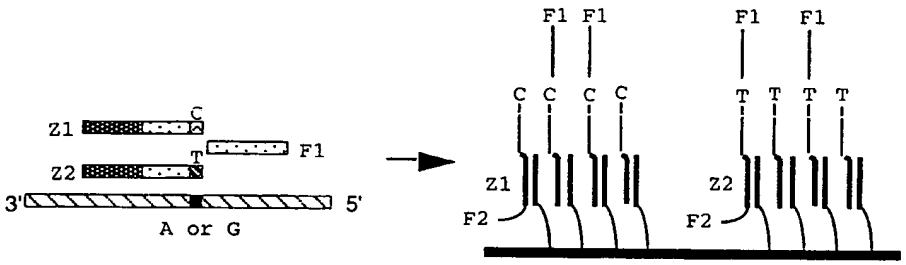


Heterozygous:
C and T alleles.

FIG. 61

PCR/LDR with Addressable Array Capture

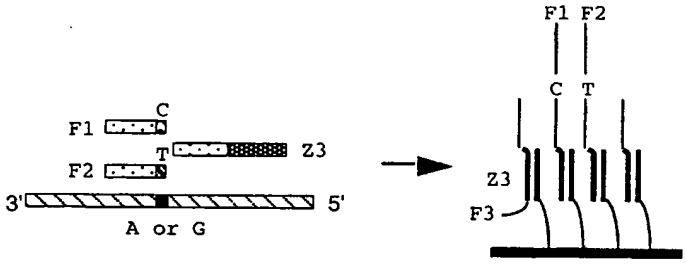
A.



LDR reaction: Zip codes on discriminating primers, small percentage labeled with F2.

Heterozygous: C and T alleles.

B.

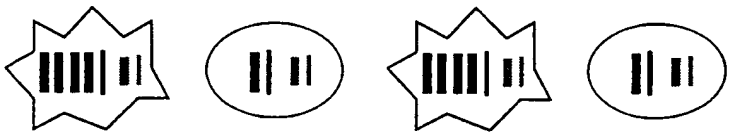


LDR reaction: Zip code on common primer, small percentage labeled with F3.

Heterozygous: C and T alleles.

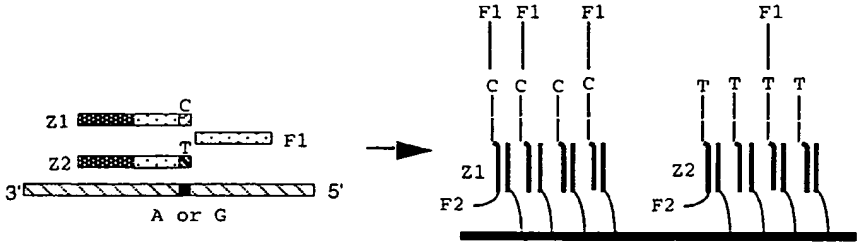
FIG. 62

PCR/LDR with Addressable Array Capture: Detection of gene amplification using zip codes on the discriminating primers.

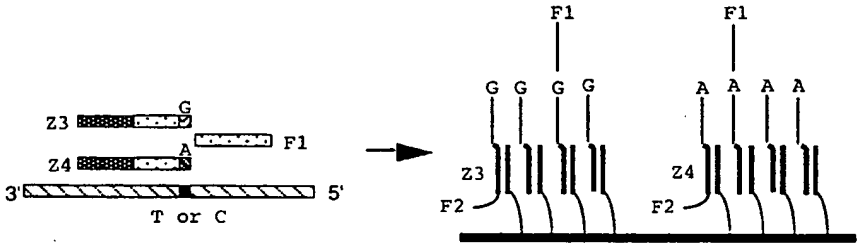


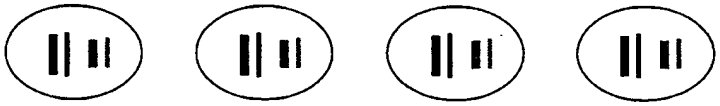
Tumor sample with 50% stromal contamination:

A. Tumor gene alleles: Ratio of C to T alleles = 10 / 4 = 2.5



B. Control gene alleles: Ratio of G to A alleles = 4 / 4 = 1.0





Normal sample with allele balance:

C. Tumor gene alleles: Ratio of C to T alleles = 4 / 4 = 1.0

D. Control gene alleles: Ratio of G to A alleles = 4 / 4 = 1.0

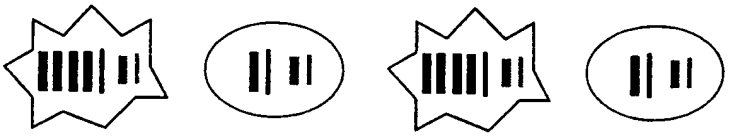
Ratio of Tumor to control allele / normal to control allele:

C : G Tumor / C : G Normal = 10 / 4 / 4 / 4 = 2.5

T : A Tumor / T : A Normal = 4 / 4 / 4 / 4 = 1.0

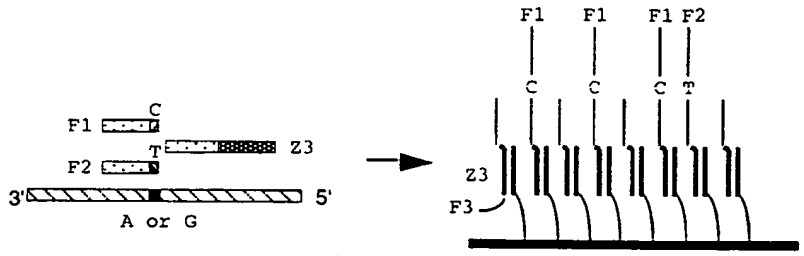
FIG. 63

PCR/LDR with Addressable Array Capture: Detection of gene amplification using zip codes on the common primers.

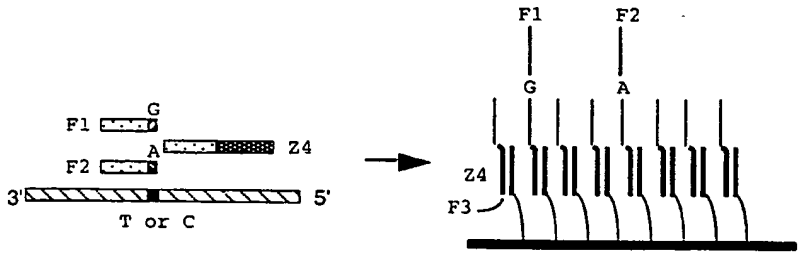


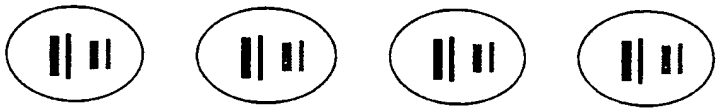
Tumor sample with 50% stromal contamination:

A. Tumor gene alleles: Ratio of C to T alleles = 10 / 4 = 2.5



B. Control gene alleles: Ratio of G to A alleles = 4 / 4 = 1.0





Normal sample with allele balance:

C. Tumor gene alleles: Ratio of C to T alleles = 4 / 4 = 1.0

D. Control gene alleles: Ratio of G to A alleles = 4 / 4 = 1.0

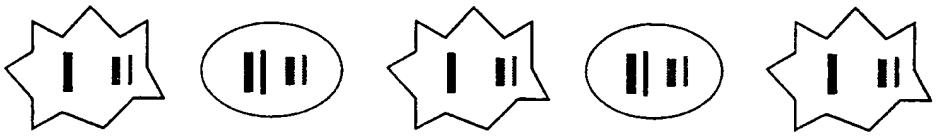
Ratio of Tumor to control allele / normal to control allele:

C : G Tumor / C : G Normal = 10 / 4 / 4 / 4 = 2.5

T : A Tumor / T : A Normal = 4 / 4 / 4 / 4 = 1.0

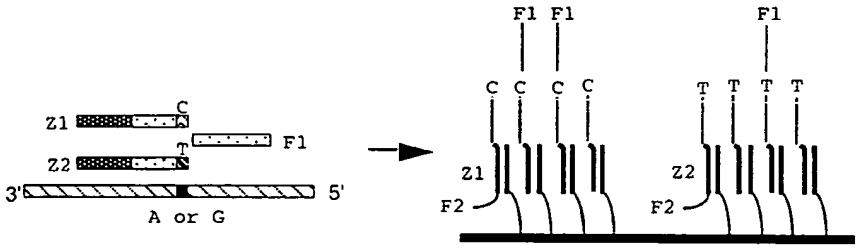
FIG. 64

PCR/LDR with Addressable Array Capture: Detection of loss of heterozygosity using zip codes on the discriminating primers.

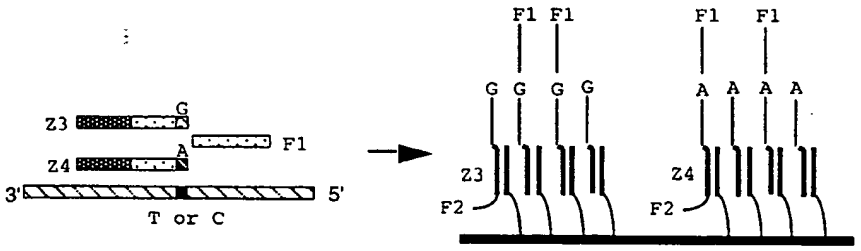


Tumor sample with 40% stromal contamination:

A. Tumor gene alleles: Ratio of C to T alleles = 5 / 2 = 2.5



B. Control gene alleles: Ratio of G to A alleles = 5 / 5 = 1.0



Normal sample with allele balance:

C. Tumor gene alleles: Ratio of C to T alleles = 5 / 5 = 1.0

D. Control gene alleles: Ratio of G to A alleles = 5 / 5 = 1.0

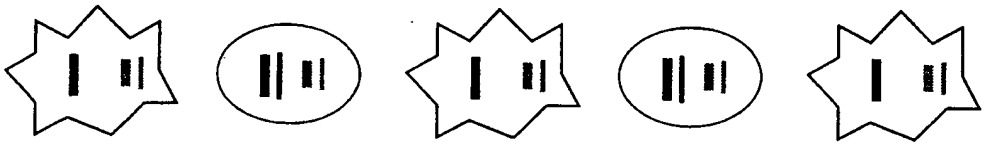
Ratio of Tumor to control allele / normal to control allele:

C : G Tumor / C : G Normal = 5 / 5 / 5 / 5 = 1.0

T : A Tumor / T : A Normal = 2 / 5 / 5 / 5 = 0.4

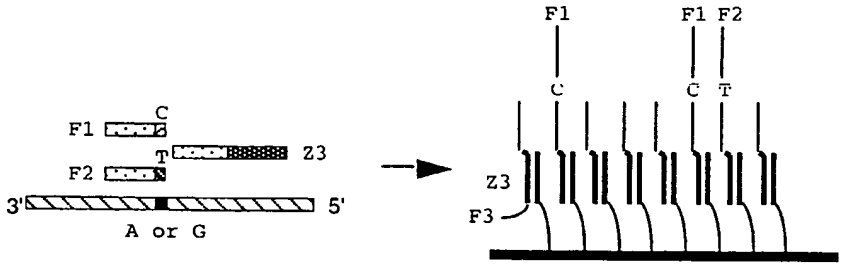
FIG. 65

PCR/LDR with Addressable Array Capture: Detection of loss of heterozygosity using zip codes on the common primers.

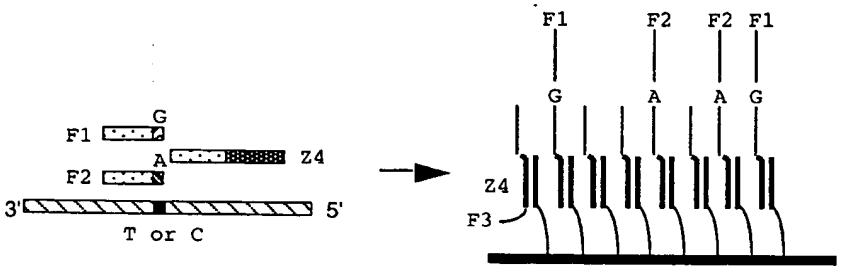


Tumor sample with 40% stromal contamination:

A. Tumor gene alleles: Ratio of C to T alleles = 5 / 2 = 2.5



B. Control gene alleles: Ratio of G to A alleles = 5 / 5 = 1.0



Normal sample with allele balance:

C. Tumor gene alleles: Ratio of C to T alleles = 5 / 5 = 1.0

D. Control gene alleles: Ratio of G to A alleles = 5 / 5 = 1.0

Ratio of Tumor to control allele / normal to control allele:

C : G Tumor / C : G Normal = 5 / 5 / 5 / 5 = 1.0

T : A Tumor / T : A Normal = 5 / 5 / 5 = 0.4

Figure 66

FIG. 66

Detection of gene amplification in tumor samples which contain stromal contamination using zip codes on the discriminating primers.

Tumor samples contains 10,000 tumor gene C alleles, and 4,000 tumor gene T alleles.

F1 for C allele (60% of 10,000)(45% capture at Z1) / F2 (10% of 100,000)(45% capture at Z1)
F1 for C allele (= 2,700) / F2 (= 4,500)
F1 for C allele / F2 = 0.60

F1 for T allele (40% of 4,000)(30% capture at Z2) / F2 (10% of 100,000)(30% capture at Z2)
F1 for T allele (= 480) / F2 (= 3,000)
F1 for T allele / F2 = 0.16

Normal samples contains 4,000 tumor gene C alleles, and 4,000 tumor gene T alleles.

F1 for C allele (60% of 4,000)(35% capture at Z1) / F2 (10% of 100,000)(35% capture at Z1)
F1 for C allele (= 840) / F2 (= 3,500)
F1 for C allele / F2 = 0.24

F1 for T allele (40% of 4,000)(50% capture at Z2) / F2 (10% of 100,000)(50% capture at Z2)
F1 for T allele (= 800) / F2 (= 5,000)
F1 for T allele / F2 = 0.16

Tumor sample contains 4,000 control gene G alleles, and 4,000 control gene A alleles.

F1 for G allele (45% of 4,000)(40% capture at Z3) / F2 (10% of 100,000)(40% capture at Z3)
F1 for G allele (= 720) / F2 (= 4,000)
F1 for G allele / F2 = 0.18

F1 for A allele (55% of 4,000)(60% capture at Z4) / F2 (10% of 100,000)(60% capture at Z4)
F1 for A allele (= 1320) / F2 (= 6,000)
F1 for A allele / F2 = 0.22

Normal sample contains 4,000 control gene G alleles, and 4,000 control gene A alleles.

F1 for G allele (45% of 4,000)(55% capture at Z3) / F2 (10% of 100,000)(55% capture at Z3)
F1 for G allele (= 990) / F2 (= 5,500)
F1 for G allele / F2 = 0.18

F1 for A allele (55% of 4,000)(45% capture at Z4) / F2 (10% of 100,000)(45% capture at Z4)
F1 for A allele (= 990) / F2 (=4,500)
F1 for A allele / F2 = 0.22

C : G Tumor / C : G Normal = (0.60 / 0.18) / (0.24 / 0.18) = 2.5
T : A Tumor / T : A Normal = (0.16 / 0.22) / (0.16 / 0.22) = 1

FIG. 67

Detection of gene amplification in tumor samples which contain stromal contamination using zip codes on the common primers.

Tumor samples contains 10,000 tumor gene C alleles, and 4,000 tumor gene T alleles.

F1 for C allele (60% of 10,000)(55% capture at Z3) / F2 (10% of 100,000)(55% capture at Z3)
F1 for C allele (= 3,300) / F2 (= 5,500)
F1 for C allele / F2 = 0.60

F1 for T allele (40% of 4,000)(55% capture at Z3) / F2 (10% of 100,000)(55% capture at Z3)
F1 for T allele (= 880) / F2 (= 5,500)
F1 for T allele / F2 = 0.16

Normal samples contains 4,000 tumor gene C alleles, and 4,000 tumor gene T alleles.

F1 for C allele (60% of 4,000)(60% capture at Z3) / F2 (10% of 100,000)(60% capture at Z3)
F1 for C allele (= 1,440) / F2 (= 6,000)
F1 for C allele / F2 = 0.24

F1 for T allele (40% of 4,000)(60% capture at Z3) / F2 (10% of 100,000)(60% capture at Z3)
F1 for T allele (= 960) / F2 (= 6,000)
F1 for T allele / F2 = 0.16

Tumor sample contains 4,000 control gene G alleles, and 4,000 control gene A alleles.

F1 for G allele (45% of 4,000)(35% capture at Z4) / F2 (10% of 100,000)(35% capture at Z4)
F1 for G allele (= 630) / F2 (= 3,500)
F1 for G allele / F2 = 0.18

F1 for A allele (55% of 4,000)(35% capture at Z4) / F2 (10% of 100,000)(35% capture at Z4)
F1 for A allele (= 770) / F2 (= 3,500)
F1 for A allele / F2 = 0.22

Normal sample contains 4,000 control gene G alleles, and 4,000 control gene A alleles.

F1 for G allele (45% of 4,000)(30% capture at Z4) / F2 (10% of 100,000)(30% capture at Z4)
F1 for G allele (= 540) / F2 (= 3,000)
F1 for G allele / F2 = 0.18

F1 for A allele (55% of 4,000)(30% capture at Z4) / F2 (10% of 100,000)(30% capture at Z4)
F1 for A allele (= 660) / F2 (=3,000)
F1 for A allele / F2 = 0.22

C : G Tumor / C : G Normal = (0.60 / 0.18) / (0.24 / 0.18) = 2.5
T : A Tumor / T : A Normal = (0.16 / 0.22) / (0.16 / 0.22) = 1

FIG. 68

Detection of loss of heterozygosity (LOH) in tumor samples which contain stromal contamination using zip codes on the discriminating primers.

Tumor samples contains 5,000 tumor gene C alleles, and 2,000 tumor gene T alleles.

F1 for C allele (60% of 5,000)(35% capture at Z1) / F2 (10% of 100,000)(35% capture at Z1)
F1 for C allele (= 1,050) / F2 (= 3,500)
F1 for C allele / F2 = 0.30

F1 for T allele (40% of 2,000)(55% capture at Z2) / F2 (10% of 100,000)(55% capture at Z2)
F1 for T allele (= 440) / F2 (= 5,500)
F1 for T allele / F2 = 0.08

Normal samples contains 5,000 tumor gene C alleles, and 5,000 tumor gene T alleles.

F1 for C allele (60% of 5,000)(30% capture at Z1) / F2 (10% of 100,000)(30% capture at Z1)
F1 for C allele (= 900) / F2 (= 3,000)
F1 for C allele / F2 = 0.30

F1 for T allele (40% of 5,000)(40% capture at Z2) / F2 (10% of 100,000)(40% capture at Z2)
F1 for T allele (= 800) / F2 (= 4,000)
F1 for T allele / F2 = 0.20

Tumor sample contains 5,000 control gene G alleles, and 5,000 control gene A alleles.

F1 for G allele (45% of 5,000)(45% capture at Z3) / F2 (10% of 100,000)(45% capture at Z3)
F1 for G allele (= 1,012) / F2 (= 4,500)
F1 for G allele / F2 = 0.22

F1 for A allele (55% of 5,000)(50% capture at Z4) / F2 (10% of 100,000)(50% capture at Z4)
F1 for A allele (= 1375) / F2 (= 5,000)
F1 for A allele / F2 = 0.27

Normal sample contains 5,000 control gene G alleles, and 5,000 control gene A alleles.

F1 for G allele (45% of 5,000)(30% capture at Z3) / F2 (10% of 100,000)(30% capture at Z3)
F1 for G allele (= 675) / F2 (= 3,000)
F1 for G allele / F2 = 0.22

F1 for A allele (55% of 5,000)(60% capture at Z4) / F2 (10% of 100,000)(60% capture at Z4)
F1 for A allele (= 1,650) / F2 (=6,000)
F1 for A allele / F2 = 0.27

C : G Tumor / C : G Normal = (0.30 / 0.22) / (0.30 / 0.22) = 1
T : A Tumor / T : A Normal = (0.08 / 0.27) / (0.20 / 0.27) = 0.4

FIG. 69

Detection of loss of heterozygosity (LOH) in tumor samples which contain stromal contamination using zip codes on the common primers.

Tumor samples contains 5,000 tumor gene C alleles, and 2,000 tumor gene T alleles.

F1 for C allele (60% of 5,000)(60% capture at Z3) / F2 (10% of 100,000)(60% capture at Z3)
F1 for C allele (= 1,800) / F2 (= 6,000)
F1 for C allele / F2 = 0.30

F1 for T allele (40% of 2,000)(60% capture at Z3) / F2 (10% of 100,000)(60% capture at Z3)
F1 for T allele (= 480) / F2 (= 6,000)
F1 for T allele / F2 = 0.08

Normal samples contains 5,000 tumor gene C alleles, and 5,000 tumor gene T alleles.

F1 for C allele (60% of 5,000)(55% capture at Z3) / F2 (10% of 100,000)(55% capture at Z3)
F1 for C allele (= 1,650) / F2 (= 5,500)
F1 for C allele / F2 = 0.30

F1 for T allele (40% of 5,000)(55% capture at Z3) / F2 (10% of 100,000)(55% capture at Z3)
F1 for T allele (= 1,100) / F2 (= 5,500)
F1 for T allele / F2 = 0.20

Tumor sample contains 5,000 control gene G alleles, and 5,000 control gene A alleles.

F1 for G allele (45% of 5,000)(40% capture at Z4) / F2 (10% of 100,000)(40% capture at Z4)
F1 for G allele (= 900) / F2 (= 4,000)
F1 for G allele / F2 = 0.22

F1 for A allele (55% of 5,000)(40% capture at Z4) / F2 (10% of 100,000)(40% capture at Z4)
F1 for A allele (= 1,100) / F2 (= 4,000)
F1 for A allele / F2 = 0.27

Normal sample contains 5,000 control gene G alleles, and 5,000 control gene A alleles.

F1 for G allele (45% of 5,000)(45% capture at Z4) / F2 (10% of 100,000)(45% capture at Z4)
F1 for G allele (= 1,012) / F2 (= 4,500)
F1 for G allele / F2 = 0.22

F1 for A allele (55% of 5,000)(45% capture at Z4) / F2 (10% of 100,000)(45% capture at Z4)
F1 for A allele (= 1,237) / F2 (=4,500)
F1 for A allele / F2 = 0.27

C : G Tumor / C : G Normal = (0.30 / 0.22) / (0.30 / 0.22) = 1
T : A Tumor / T : A Normal = (0.08 / 0.27) / (0.20 / 0.27) = 0.4

FIG. 70

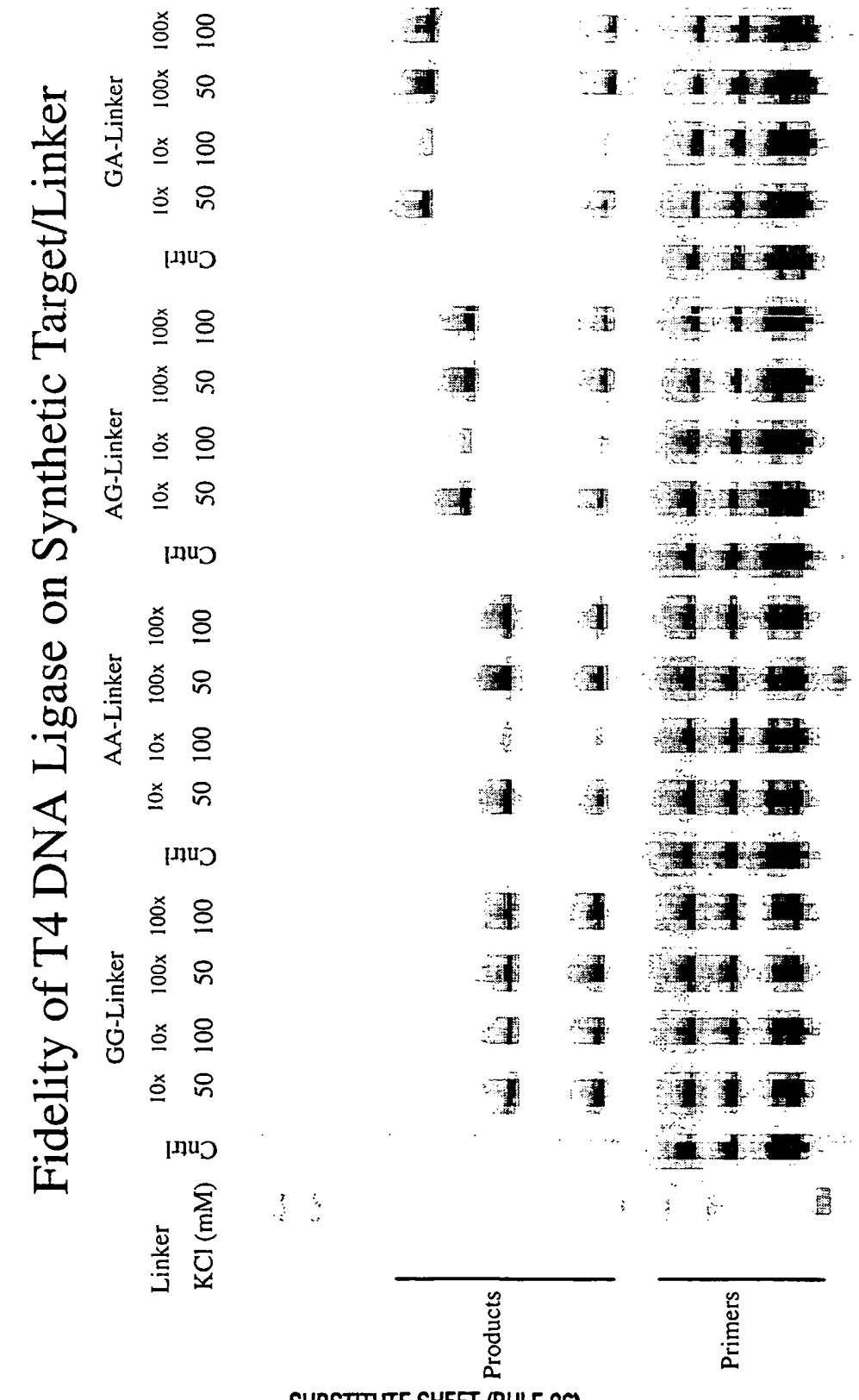

Fidelity of T4 DNA Ligase on Synthetic Target/Linker
Linker
KCl (mM)
GG-Linker
AA-Linker
AG-Linker
GA-Linker
Cntrl
10x 10x 100x 100x
50 100 50 100
Products
Primers
TC
CT
TT or CC
Common Product
TT or CC Common

FIG. 71

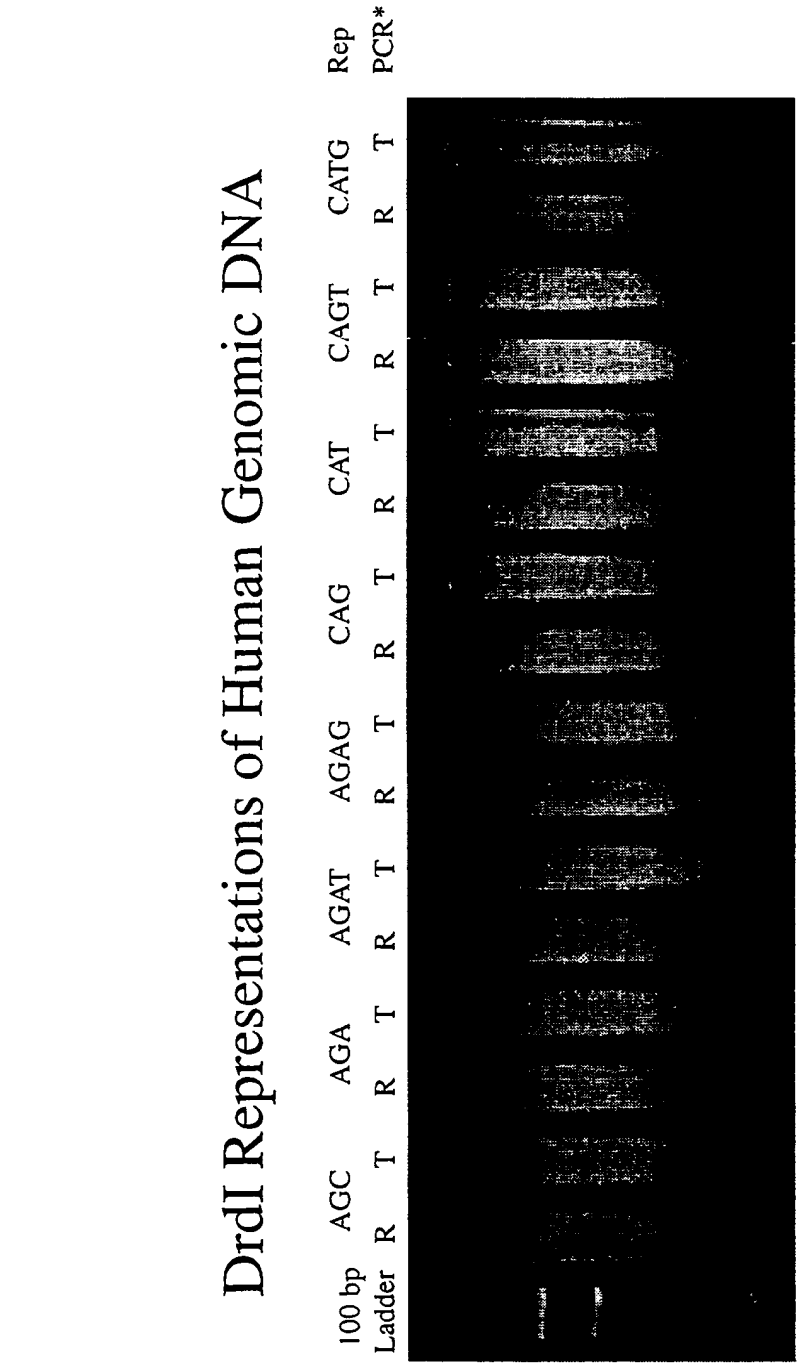

DrdI Representations of Human Genomic DNA
100 bp Ladder
AGC R T
AGA R T
AGAT R T
AGAG R T
CAG R T
CAT R T
CAGT R T
CATG R T
Rep PCR*
* R = regular T = Touchdown

FIG. 72

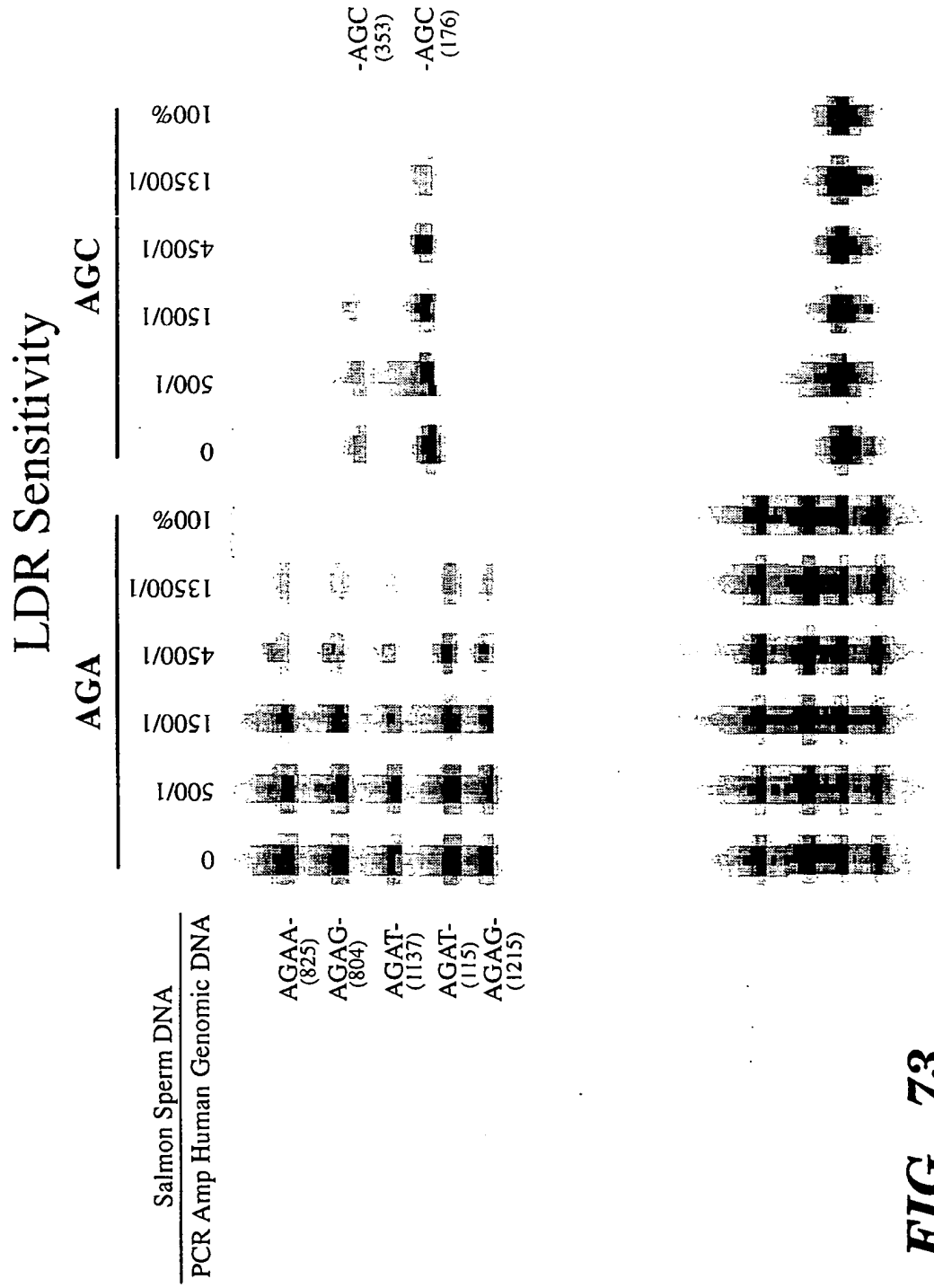

LDR Sensitivity
AGA
AGC
0
500/1
1500/1
4500/1
13500/1
100%
AGAA-(825)
AGAG-(804)
AGAT-(1137)
AGAT-(115)
AGAG-(1215)
-AGC (353)
-AGC (176)
Salmon Sperm DNA
PCR Amp Human Genomic DNA

FIG. 73

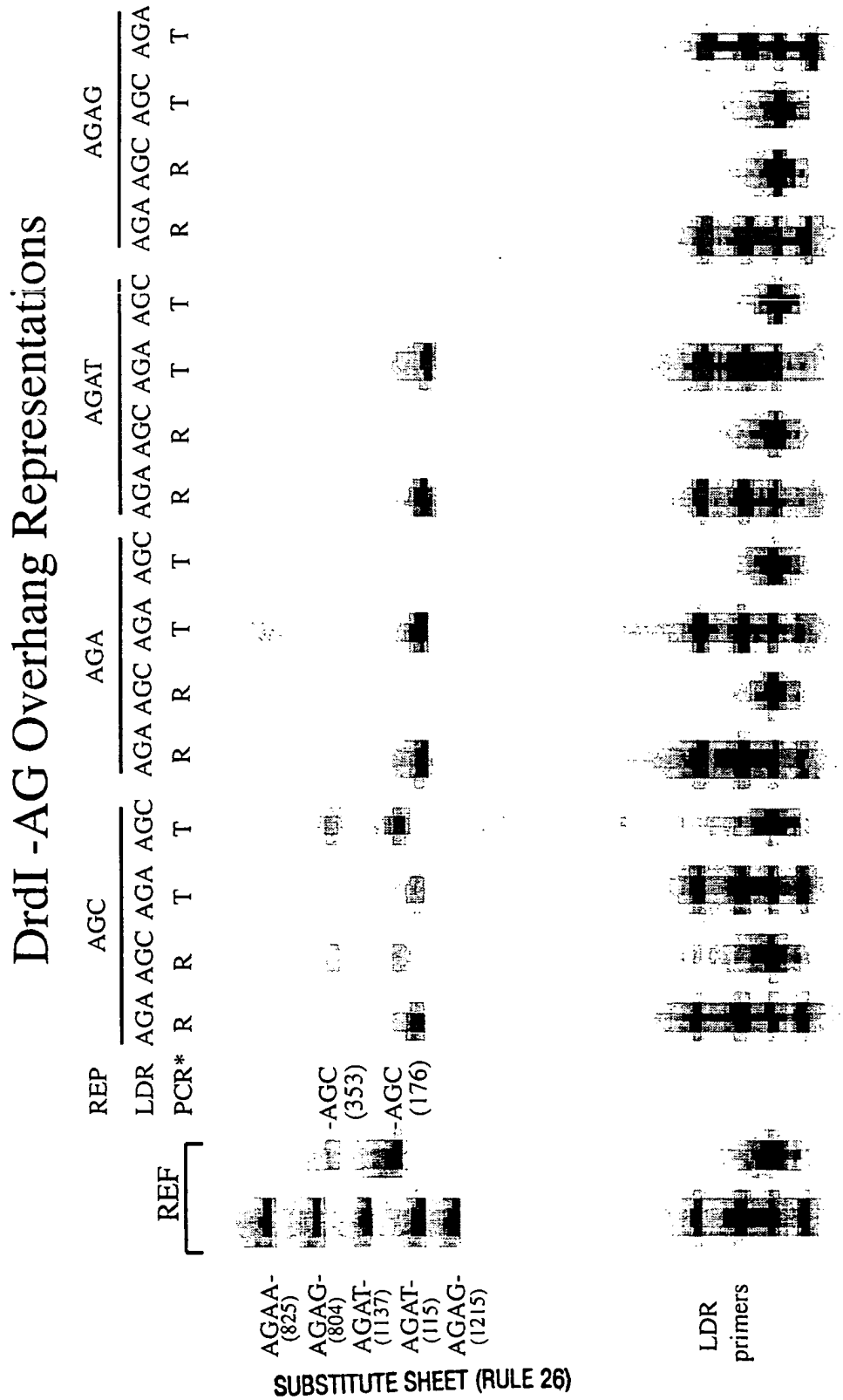

DrdI -AG Overhang Representations
REP
LDR
PCR*
REF
AGC
AGA
AGAT
AGAG
AGA AGC AGA AGC AGA AGC AGA AGC AGA AGC AGA AGC AGA AGC AGA AGC
R R T T R R T T R R T T R R T T
-AGC (353)
-AGC (176)
AGAA- (825)
AGAG- (804)
AGAT- (1137)
AGAT- (115)
AGAG- (1215)
LDR primers
* R = regular T = Touchdown

FIG. 74

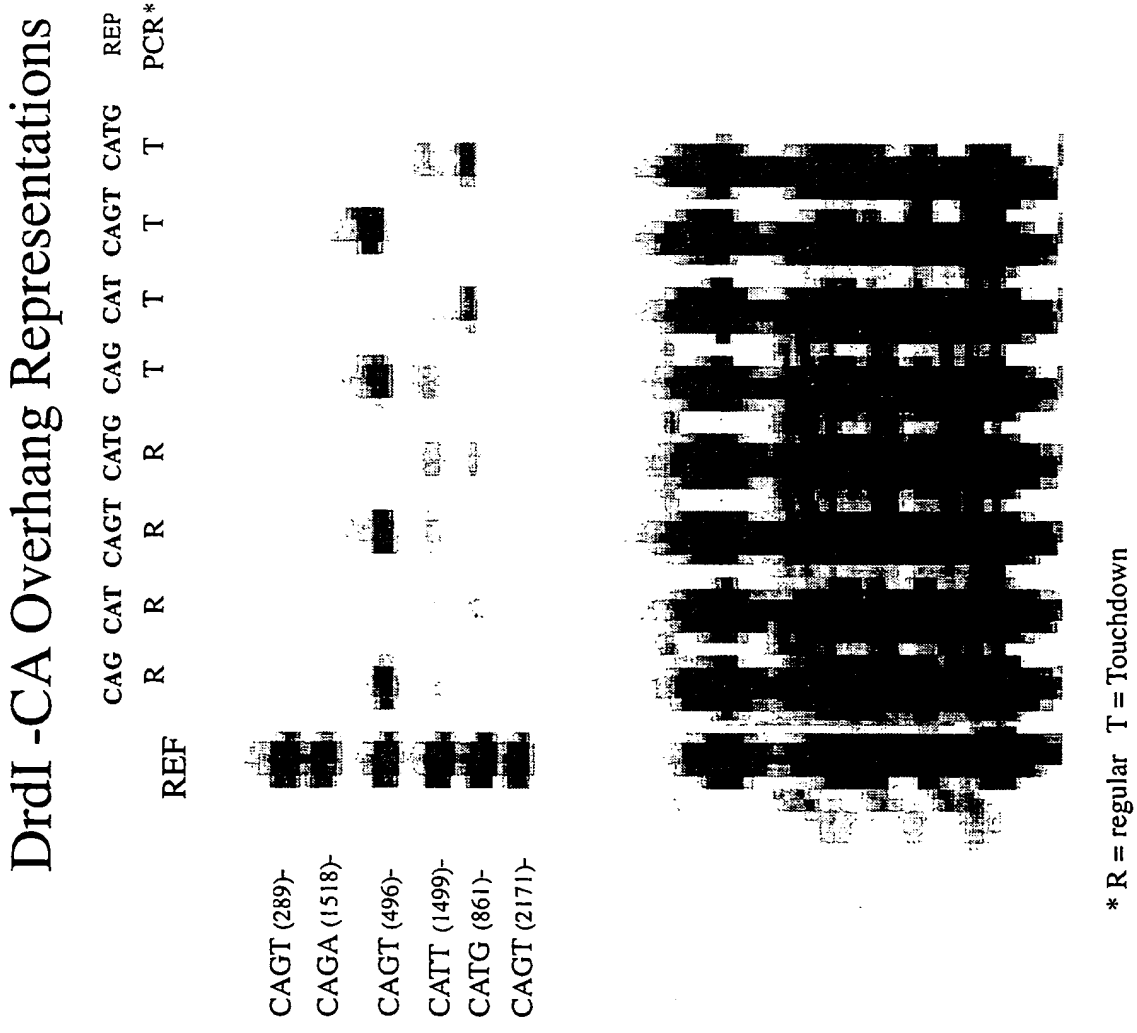

DrdI -CA Overhang Representations
REF
CAG R
CAT R
CAGT R
CATG R
CAG T
CAT T
CAGT T
CATG T
REP PCR*
CAGT (289)-
CAGA (1518)-
CAGT (496)-
CATT (1499)-
CATG (861)-
CAGT (2171)-
* R = regular T = Touchdown

FIG. 75

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(25) Filing Language: English

(26) Publication Language: English

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(74) Agents: GOLDMAN, Michael, L. et al.; Nixon Peabody LLP, Clinton Square, P.O. Box 1051, Rochester, NY 14603 (US).

(81) Designated States *(national)*: AU, CA, JP.

(84) Designated States *(regional)*: European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published:
— *With international search report.*

(88) Date of publication of the international search report:
4 January 2001

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 00/40755 A3

(54) Title: METHOD FOR ACCELERATING IDENTIFICATION OF SINGLE NUCLEOTIDE POLYMORPHISMS AND ALIGNMENT OF CLONES IN GENOMIC SEQUENCING

(57) Abstract: The present invention is directed to a method of assembling genomic maps of an organism's DNA or portions thereof. A library of an organism's DNA is provided where the individual genomic segments or sequences are found on more than one clone in the library. Representations of the genome are created, and nucleic acid sequence information is generated from the representations. The sequence information is analyzed to determine clone overlap from a representation. The clone overlap and sequence information from different representations is combined to assemble a genomic map of the organism. Once the genomic map is obtained, genomic sequence information from multiple individuals can be applied to the map and compared with one another to identify single nucleotide polymorphisms. These single nucleotide polymorphisms can be detected, and alleles quantified, by conducting (1) a global PCR amplification which creates a genome representation, and (2) a ligation detection reaction process whose ligation products are captured by hybridization to a support.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 00/00144

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12Q1/68 //C12N15/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12Q C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	EP 0 593 095 A (GENENTECH INC) 20 April 1994 (1994-04-20) claims 1-14 ---	1-3 4-87
X Y	WO 98 46621 A (BRENNER SYDNEY ;DUBRIDGE ROBERT B (US); GRYAZNOV SERGEI M (US); LY) 22 October 1998 (1998-10-22) the whole document ---	1-3 4-87
Y	GB 2 295 228 A (UNILEVER PLC) 22 May 1996 (1996-05-22) claims 1-8 ---	1-87
Y	WO 98 10095 A (BRAX GENOMICS LTD ;THOMPSON ANDREW HUGIN (GB); SCHMIDT GUNTER (GB)) 12 March 1998 (1998-03-12) the whole document ---	1-87
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
3 July 2000	04 10 2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	OSBORNE, H

3

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 00/00144

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-87 (complete)

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

1. Claims: 1-87

Methods for the assembling genomic maps of an organism's DNA or portions thereof, and methods for identifying single nucleotide polymorphisms fom said assembled maps.

2. Claims: 88-90

A method to sequence directly from a PCR amplified nucleic acid without primer interference.

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Information on patent family members

International Application No
PCT/US 00/00144

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